# 05-31/06-01 pSVA1 and pRS415-GAL plasmid amplification

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-05-31 to 2016-06-01

## TUESDAY, 5/31/16

Plasmids were amplified to be sent to GeneArt for the cloning of our microcystinase constructs.

### Transformation

TOP10 *Escherichia coli* competent cells were thawed on ice for 30 min. 50 ul of thawed competent cells was mixed with plasmid DNA; 1 ul (15,1 ng) of pSVA1, 0,5 ul of pRS415-GAL.
Mixture was incubated on ice for 30 min, then heat shocked in a 42 C water bath for 45 seconds, followed by 2 min of incubation on ice. 500 ul of LB medium was added to each competent cell/plasmid mixture. Cells were grown for 45 min in a shaking incubator at 37 C, 250 rpm.
After outgrowth step, approximately half of each transformation mixture was spread on four plates containing antibiotics (ampicillin for pRS415-GAL, kanamycin for pSVA1), and plates were incubated overnight at 37 C.

## WEDNESDAY, 6/1/16

### Liquid culture

Two 50 ml eppendorf tubes containing 5 ml of LB medium + antibiotics (50 ug/mL kanamycin in one, 100 ug/mL ampicillin in the other) were inoculated from plates of transformation mixture grown overnight; pSVA1 in the tube containing kanamycin, pRS415-GAL in the one containing ampicillin. Cells were grown for 8 hours in a shaking incubator at 37 C, 240 rpm.

### Plasmid purification

Plasmid DNA was extracted and purified from the grown cells using a plasmid purification kit (NucleoSPin Plasmid, Machery-Nagel) and the protocol provided with the kit. Plasmid DNA concentration was measured from the buffer solution containing purified plasmid DNA and the solution was stored in -20 C.

# 07-11/20 Expression of mlrA in E. coli

**Project:** Microcystinase (MlrA)
**Authors:** Snehadri Sinha
**Dates:** 2016-07-10 to 2016-07-20

SUNDAY, 7/10

We will express mlrA enzyme in E. coli as a positive control for the actual expression in yeast cells later. We will also test if 10% glycerol makes any difference in the expression, since it should help with production of difficult proteins. The expressed enzymes will be taken to Viikki to Kaarina Sivonen's group for enzyme activity analysis.

MONDAY, 7/11

Transformation of mlrA into E. coli BL21(DE3) cells

## Materials

E. coli BL21(DE3) One Shot competent cells

## Work flow

**Transformation**

- Tranformation according to One Shot transformation protocol
    - 10 ng of DNA to cells (MlrA in pSVA1 plasmid, synthesized by GeneArt, suspended in water in a concentration of 100 ng/uL -> 1 uL of this used)
    - 20 ul, 50 ul, 100 ul, ~ 80 ul were plated on LB-kan plates

TUESDAY, 7/12

Primary cultures for expression

## Materials

Kanamycin stock 50 mg/ml

LB-kan 10% glycerol

- 4 mL LB + 50ug/ml kan
- 1 mL 50% glycerol

LB-kan

- 5 ml LB with 50 ug/ml kan

## Work flow

**Transformation**

- Best colonies on plate with 50 ul bacteria plated, so this was chosen for the primary cultures

**Primary cultures**

- Single colony inoculated in 5 ml of LB-kan
    - Two samples in LB with 10% glycerol, two without
- Primary cultures O/N, at +37 °C, shaking 230 rpm

WEDNESDAY, 7/13

Protein expression induction

## Work flow

**Protein expression**

- Media in primary cultures appeared cloudy, suggesting bacterial growth

- 2 ml of primary culture to 50 ml of LB with 50 ug/ml kan
    - Glycerol samples with 10 % glycerol
- Incubation at +37 °C, shaker 230 rpm, started at 10:00
- OD values in Table 1
    - the culture with glycerol grew considerably slower than without
    - the other culture without glycerol reached OD of 1,11 before induction

Table1

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Sample | 11:45 | 12:30 | 13:30 | 13:55 | 14:15 | 15:00 |
| 2 | MlrAE 10% glycerol | | 0.16 | 0.35 | | 0.448 | |
| 3 | MlrAE 10% glycerol | | | 0.37 | | 0.438 | |
| 4 | MlrAE no glycerol | 0.06 | 0.18 | 0.65 | 0.87 | | |
| 5 | MlrAE no glycerol | | | 1.11 | | | |

- 1 ml of samples were taken for SDS-PAGE as the before induction samples
    - Samples centrifuged at 10 000 g for 5 min, then 3 min, pellet was stored at -20 °C
- Induction with 1 mM IPTG
- Expression at 22 °C, shaking, O/N, started at 15:30

THURSDAY, 7/14

Expression continues...

## Work flow

**Protein expression**

- 11:30 am cultures taken out from shaker
- 1 ml from each flask taken for SDS-PAGE as after induction sample
    - Centrifuged at 5 000 g for 8 min
    - Pellet and supernatant stored separately at -20 °C for analysis
- Remaining culture into 50 mL falcon tubes, centrifuged at 3 900 g for 20 min at +4 °C
- Supernatant discarded, pellets stored at -20 °C

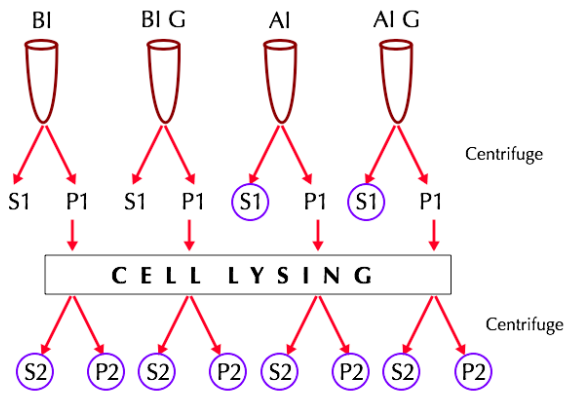
FRIDAY, 7/15

Preparing samples for SDS-PAGE analysis.
SDS-PAGE will be analyzed with Coomassie blue staining and Western blot.

## Samples and flowchart

**BI** = before induction
**AI** = after induction
**S** = supernatant
**P** = pellet
**G** = expression with 10% glycerol

Flow chart.png




SDS-PAGE samples circled

## Work flow

**Cell lysing with glass beads, pellet-1 samples**

- BI-P1, BI-P1G, AI-P1, AI-P1G suspended in dd$H_20$
- Centrifuged at 5 000 g for 5 min at +4 °C
- Pellets were combined and resuspended in SDS-PAGE sample buffer and lysed with glass beads, by vortexing at full speed for 10 min
    - 0.25 M DTT was added to SDS-PAGE sample buffer just before resuspension
- Samples were centrifuged at 20 000 g for 5 min at +4 °C
- Supernatant was collected, boiled at 70 °C for 5 min, then stored at -20C
    - Pellets were mixed with the glass beads, wasn't able to collect it, so no BI-P2, BI-P2G, AI-P2, AI-P2G

**Acetone precipitation, supernatant-1 samples**

- AI-S1, AI-S1G mixed with 10 mL acetone
- Precipitation by incubating at -20C for 30 min
- Samples centrifuged at 12 000 g for 5 min at +4 °C
- Supernatant was decanted and samples were air-dried for acetone to evaporate
- 15 ul of SDS-PAGE sample buffer was added to tubes and combined (~ 90 ul total volume for each sample)
- Samples boiled at +95 °C for 5 min and then stored at -20 °C

MONDAY, 7/18

Preparing SDS-PAGE samples with sonication.
We had to prepare SDS-PAGE samples for BI-P2, BI-P2G, AI-P2, AI-P2G since we were unable to get them with glass bead cell lysis (check 15.7.).
Coomassie blue staining and western blot.

## Materials

Bacterial samples from 50 mL cultures from 13.5.

Lysis buffer

- PBS
- Roche cOmplete protein inhibitor mix tablet

PBS-T (0,1% Tween-20)

- 50 mL 20x PBS-T
- 950 mL H2O

Transfering buffer

- 10 mL 10x stock
- 20 mL methanol
- 70 mL H2O

5% (w/v) milk for blocking

- 0,75 g milk powder
- 15 mL PBS-T

1 : 5 000 primary Ab in 3% (w/v) BSA

- 0,45 g BSA
- 15 mL PBS-T
- 3 ul anti-His antibody

## Work flow

**Sample preparation by sonication**

- Resuspended bacterial expression pellets to 5 mL lysis buffer
- Added small amount (1/4 of small spatula) of lysozyme to each sample
- Sonication with tip sonicator on ice 5 sec on/5 sec off for 5 min, smallest tip, 20 % amplitude
- Centrifuge in eppendorf tubes 15 min, 15 000 rpm, +4 °C
- Resuspended pellets to 1,5 mL of PBS
- Prepared SDS-PAGE samples
    - 25 ul 4xLD + 75 ul sample
    - boiled +70 °C, 5 min

**SDS-PAGE**

- Samples were loaded on two gels
    - Pellet-2 samples were really viscous
- Started running at 80 V 1h, increased to 120 V until ready
- One gel to Coomassie blue staining
    - staining 1h RT shaking
    - destaining O/N shaking RT

**Western blot**

- Blotting with TURBO transfer (standard settings) - 7 min
- Blocking with 5% milk, 1,5 h, shaking, RT
- Rinsed 3x, washed 2x 4 min in shaking with PBS-T
- Primary antibody O/N, shaking, +4 °C

TUESDAY, 7/19

Results from SDS-PAGE Coomassie blue and WB.

## Materials

1 : 30 000 secondary Ab in 1% (w/v) milk

- 0,15 g milk powder
- 15 mL H2O
- 0,5 ul anti-anti-His antibody

## Work flow

**SDS-PAGE, Coomassie**

- Imaged the destained gel

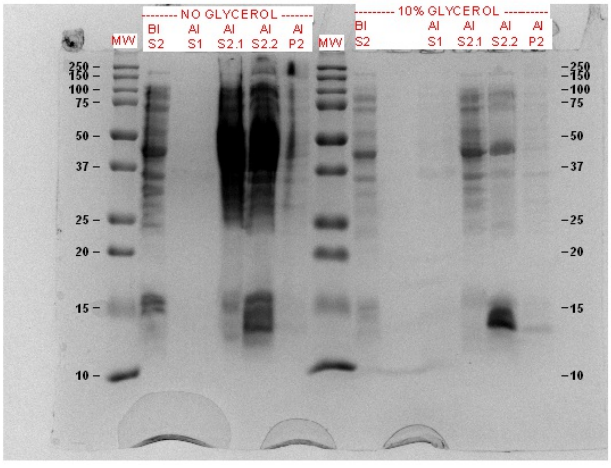
**Western blot**

- Collected primary antibody for future use
    - added small amount of NaN3 to preserve it (good for upto 10 uses)
    - stored in +4 °C
- Washing step: 2x rinse + 3x 5 min wash shaking with PBS-T
- Secondary antibody incubation 1 h, RT, shaking
- Washing as earlier
- Substrate solution prepared 2 mL 1:1 and pipeted to membrane
- 1 min incubation
- Imaging
- Washing as earlier
- Stored in a box, RT, dried, if there is a need for a new analysis

## Results and discussion

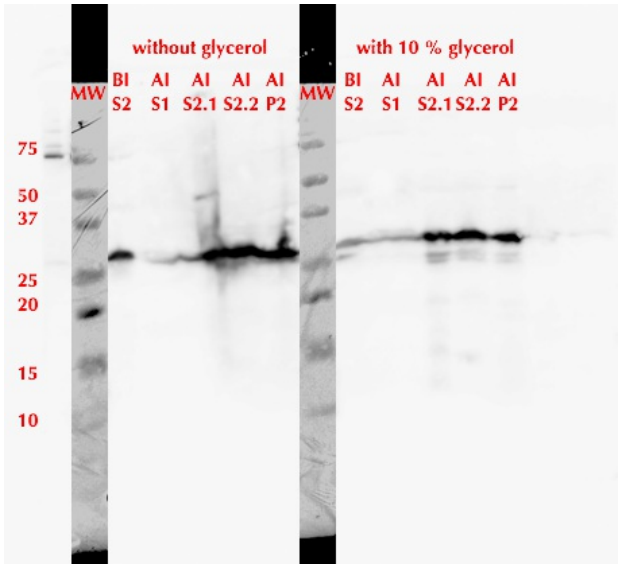
**Coomassie blue**

2016-07-19 mlrA, ecoli.tif




**Western blot**

MlrA in E. coli with and without glycerol




BI = before induction
AI = after induction
S1 = sup after expression culture is spinned down
P2 = pellet after cell lysis with sonication and spin
S2.1 = sup after cell lysis with glass beads and spin
S2.2 = sup after cell lysis with sonication and spin

On the Coomassie blue gel it seems that there is no apparent overexpression of any protein.
In the western blot there is a strong signal from a band that is about 28 kDa, which is the expected size. This band is best seen in AI-S2.1, AI-S2.2 and AI-P2 samples. There is also quite clear band in BI sample without glycerol. This could mean that our promoter is leaky?
In BI sample with glycerol there is also this band. Strong bands in S2.1 and S2.2 indicate that protein is soluble. There is though also a strong band in pellet sample suggesting that some of the protein has remained in membranes or formed inclusion bodies.

We decided to give the AI-S2.2 samples to enzyme activity test in Viikki.

Using 10 % glycerol in the growing media slowed the growth rate which led to smaller amount of enzyme.

## WEDNESDAY, 7/20

We should have incubated the samples with MC extract for the analysis, so this will be done later.

The plan now is to express more mlrA and purify it with His-tag so we get a bit purer sample, and do samples with that for the enzyme activity analysis.

# 07-13 SDS-PAGE

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-07-13 to 2016-08-05

## WEDNESDAY, 7/13

Following solutions were prepaired:

- 1,5M Tris-HCl pH 8,8
- 1M Tris-HCl pH 6,8
- 0,25M Tris-HCl pH 6,8

How?

1. measure 9,086g/6,07g/1,514g of Tris
2. dilute into 40ml of water
3. adjust pH
4. add water to 50 ml

## THURSDAY, 7/14

Following solutions were prepared:

4xSeparating gel buffer (1,5M Tris-HCl pH 8,8, 0,4% SDS)
2xStacking gel buffer (0,25M Tris-HCl pH 6,8, 0,2% SDS)
10% APS (0,1 g +1 ml of water)
5x Sample running buffer with following instructions:

Table1

| | A | B | C |
|---|---|---|---|
| 1 | component | amount | composition of working solution |
| 2 | 1M Tris-HCl pH 6,8 | 2,25ml | 0,225M |
| 3 | 99,5% glycerol | 5ml | 50% |
| 4 | SDS | 0,5g | 5% |
| 5 | Bromophenol blue | 5mg | 0,05% |
| 6 | 1M DTT (needs to be added right before use) | 2,5ml | 0,25M |

- sample buffer was stored in freezer. 1M DTT needs to be added when used.

Acrylamide gels were prepaired using attached casting SDS-PAGE gel protocol.

## TUESDAY, 7/26

4 x 12.5% acrylamide gels prepared.

4 x 12.5% acryalmide gels (separating)

| | A | B |
|---|---|---|
| 1 | Component | Volume |
| 2 | 1.5 M Tris-HCl (separating buffer) | 5 mL |
| 3 | H2O | 8.75 mL |
| 4 | 40% acrylamide | 6.3 mL |
| 5 | 10% APS | 120 uL |
| 6 | TEMED | 25 uL |

4 x 3% acrylamide gels (stacking)

| | A | B |
|---|---|---|
| 1 | Component | Volume |
| 2 | 0.25 M Tris-HCl (stacking buffer) | 3.8 mL |
| 3 | H2O | 3.2 mL |
| 4 | 40% acrylamide | 0.6 mL |
| 5 | 10% APS | 60 uL |
| 6 | TEMED | 12.5 uL |

Only one 12.5% SDS-PAGE gel was prepared and stored at +4C. Other gels leaked! --> Issue lies with stacking the glass plates together.
TIP: When aligning plates they should be done inside the green holder and then altogether inserted between the clip and the rubber base.

WEDNESDAY, 7/27

12.5% acrylamide gels were prepared with same conditions as yesterday. Alignment was done properly.
4 gels were made and stored in +4C.

FRIDAY, 8/5

4 x 12.5% acrylamide gels were prepared and stored in +4C.

# 07-20/29 Expression and purification of mlrA in E. coli

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-07-20 to 2016-07-29

WEDNESDAY, 7/20

Based on the earlier expression of mlrA in E. coli (07-11/20 Expression of mlrA in E. coli), we decided to express more mlrA with just one kind of condition for the enzyme activity test, but now we will purify the enzyme a bit further.

## Materials

E. coli BL21(DE3) with mlrA in pSVA1 plasmid, transformed 11.7.

LB + 50 ug/ml kan

## Work flow

**Primary cultures**

- Single colonies where incubated in 5 mL of LB + kan
- O/N, at +37 °C, shaking

THURSDAY, 7/21

Induction of mlrA expression

## Work flow

**Primary cultures**

- Tubes were taken from incubator in the morning and kept at room temperature.
- O.D. of O/N cultures were 0.645 and 0.737.

**Expression cultures**

- 0.5 mL of overnight culture was added to each flask containing 50 mL LB + 50 ug/ml kan. Cultures from one colony went to two flasks.
- Flasks were incubated at +37 °C at 12:30. O.D. was measured at regular intervals.

Table1

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Time | 1 | 2 | 3 | 4 |
| 2 | 14:00 | 0.021 | 0.02 | 0.039 | -0.072* |
| 3 | 15:30 | 0.162 | 0.135 | 0.334 | 0.303 |
| 4 | 16:00 | | | 0.626 | 0.59 |
| 5 | 16:20 | | | 0.92 | 0.9 |
| 6 | 16:30 | 0.704 | 0.667 | | |
| 7 | 16:40 | 0.879 | 0.888 | | |

* After low O.D. was reported for flask 4 at 14:00, 0.5 mL of O/N culture was again added to the medium.

- 1 mL of culture was taken from flasks 2 and 4 as pre-induction (BI) samples for SDS-PAGE analysis. It was centrifuged at 10000 g for 5 min. Supernatant was discarded and pellet was stored in -20C.
- 1 mM IPTG (250 ul) was added to each flask to induce protein production. Flasks were incubated at +22C O/N. For first 15 minutes of incubation, temperature was at +32C (did not go lower in that time).

FRIDAY, 7/22

- Flasks were taken out of shaker incubator around 13:10
- 1 mL culture was taken from flasks 2 and 4 as post-induction (AI) samples for SDS-PAGE analysis
    - Centrifuged at 10000 g for 5 min
    - Supernatant and pellet stored separately at -20C
- Remaining culture was centrifuged at 3900 g for 20 min at +4C. Supernatant was discarded and pellets were stored at -20C.

MONDAY, 7/25

## Materials

Buffers for Ni-NTA purification

- Equilibration buffer (EQB)
    - PBS + 10 mM imidazole, pH 7.4
- Washing buffer (WB)
    - PBS + 25 mM imidazole, pH 7.4
- Elution buffer (ELB)
    - PBS + 250 mM imidazole, pH 7.4

Lysis buffer

- PBS + protein inhibitor mix
    - from 18.7. which was stored in -20 °C

## Work flow

Buffers for Ni-NTA purification

- made 5 ml of ELB and used that as a 25x and 10x stock for EQB and WB, respectively
- measured 87 mg of imidazole, suspended in PBS, adjusted pH to 7.4
- EQB: 0,2 ml ELB + 4,8 ml PBS, checked pH
- WB: 0,3 ml ELB + 2,7 ml PBS, checked pH

Cell lysis with sonication

- Cell pellet from 200 mL culture was resuspended into 10 mL lysis buffer
- Sonication with the smallest tip, amplitude 20 %, 5 sec on / 5 sec off
- Centrifugation 15 min, 15 000 g, +4 °C
- Supernatant and pellet were stored separately in -20 °C

TUESDAY, 7/26

## Materials

For Ni-NTA resin regeneration

- 20 mM MES buffer, 0.1 M NaCl, pH 5.0
- 20% ethanol

Codes for samples

- FT = Flow through fraction
- W = Washing fraction
- E = Elution fraction

## Work flow

Ni-NTA purification according to protocol from Thermo Scientific (https://tools.thermofisher.com/content/sfs/manuals/MAN0011699_HisPur_NiNTA_SpinColumn_UG.pdf), with these changes/remarks

- everything was done at +4 °C or on ice
- loaded the column 5 times (volumes in table 2)
    - weren't sure how much the column would bind, so we decided to over-load it and then run the flow throughs on the gel to see at which point the column didn't bind our protein anymore
    - after the second loading (FT2) the sample incubated about 40 min in the mixer
    - after collecting FT4 the Ni-NTA resin was incubated about 40 min without any sample -> hopefully didn't dry out, load 5 was added and incubated normally

Table2

| | A | B | C |
|---|---|---|---|
| 1 | Load | sample 1 | sample 2 |
| 2 | 1 | 800 ul | 700 ul |
| 3 | 2 | 500 ul | 500 ul |
| 4 | 3 | 500 ul | 500 ul |
| 5 | 4 | 500 ul | 500 ul |
| 6 | 5 | 500 ul | 500 ul |

- after elutions we did regeneration of the spin columns

Resin regeneration

- Prepared MES buffer
    - measured 19,52 mg of MES hydrate and 29,22 mg of NaCL, suspended in 5 mL water
    - adjusted pH to 5.0
- Each column washed with 2 mL MES buffer at 700 g for 2 min
- Wash with 2 mL H20, same centrifuging conditions
- 200 ul 20 % ethanol added to resin
- Tubes stored in +4 °C

SDS-PAGE sample preparation

- All the samples for the gel were
    - BI-S2, BI-P2, AI-S1, AI-S2, AI-P2 from 22.7. and 25.7.
        - The AI-P1 sample had to be lysed to get the AI-S2 and AI-P2 samples, cell lysis with sonication, resuspended the pellet into 2 mL lysis buffer (PBS + protein inhibitor mix), didn't add lysozyme, sonication as earlier (25.7.), centrifuge 7 min, 15 000 g, RT
    - FT1-5, W1-3, E1-4 from today, each from sample 1 and 2
    - altogether 29 samples
- 12,4 ul 4xLD + 37,6 ul sample
- stored to -20 °C

WEDNESDAY, 7/27

## Materials

Transfer buffer, 100 mL

- 20 ml of 5 x running buffer (this is running buffer for SDS-PAGE)
- 20 ml metanol
- 60 ml H2O

PBS-T, 1 L

- 50 mL of 20x stock
- 950 mL of H2O

Blocking solution, 3% BSA, 15 mL

- 0,45 g of BSA
- 15 ml PBS-T

PBS-T, 20x stock, pH 7.6

- 14.4 g $Na_2HPO_4$
- 80 g NaCl
- 2.4 g $KH_2PO_4$
- 2 g KCl
- 5 mL Tween 20
- Made up to 500 mL with $H_2O$
- pH adjusted to 7.6 with 4M NaOH

## Work flow

SDS-PAGE

- Boiled samples from yesterday at +70 °C, 5 min
- Loaded two identical gels, one for Coomassie blue staining, other for Western blot
    - started running at 80 V at 10:10
    - increased voltage to 120 V at 10:45

Coomassie blue staining

- Gel was put to staining 2 h, RT, shaking
- Destain O/N, RT, shaking, started at 14:10

Western blot

- Transfer buffer, PBS-T and blocking solution were prepared
- Blotting was done according to western blot protocol
- Membrane put to blocking with 3 % BSA in PBS-T, 1,5 h, shaking, RT
- Primary antibody in 3 % BSA in PBS-T from last time (18.7.) was added and left O/N, +4 °C, shaking at 14:07

THURSDAY, 7/28

## Materials

Secondary antibody solution

- 1 % milk in PBS-T with 1 : 30 000 dilution of secondary antibody (anti-anti-His)
- 0.5 ul antibody in 15 ml 1 % milk in PBS-T

## Work flow

Coomassie blue staining

- Imaged gels (see results)

Western blot

- Made 1% milk in PBS-T, added 0.5 ul of secondary antibody
    - 1 : 30 000 dilution
- Washing step: rinse twice, 3 x 5 min wash in shaking, all with PBS-T
- Added secondary antibody solution, 1h, RT, shaking
- Washing step as earlier
- Detection solution 1 + 1 ml, imaged (see results)
- Membrane was washed and stored dried, RT, in a box

SDS-PAGE

- Ran rest of the samples from yesterday on two identical gels (one for coomassie, other for WB)
    - started with 80 V, increased at some point to 100 V
- One gel to coomassie blue staining, 1 h, RT, shaking and then destaining O/N
- Other gel transfered to nitrocellulose membrane with 30 min programme (to see if there is less protein left on the gel after), WB according to protocol attached uptill primary antibody
    - Gel from transfer to stain O/N, RT, shaking

## Results and conclusions

We had saved the images from this in the wrong formate -> were unable to get them open

FRIDAY, 7/29

Results from yesterday's blots

## Work flow

Coomassie blue
We had two gels, one proper one and then gel from WB to see if there was protein left in the membrane

- Changed the WB left over gel into destain
- Imaged gels (see results)

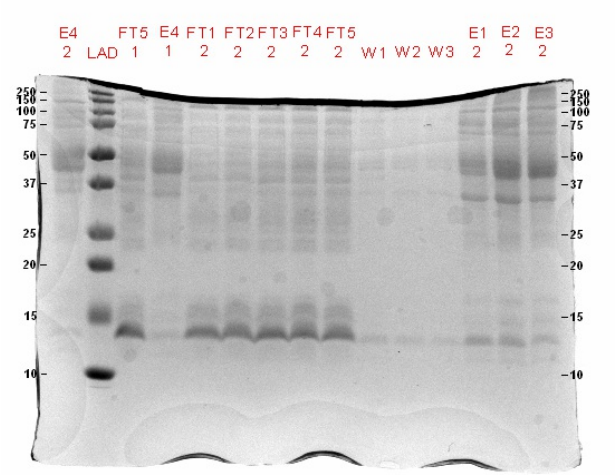
Western blot

- Collected primary antibody solution
    - The primary antibody was left ON in RT, so it might be that it has degraded or damaged somehow, or that there is a lot of non-specific binding on the membrane
- Rest of WB as the attached protocol says
- Imaged membrane (see results)

## Results and conclusions

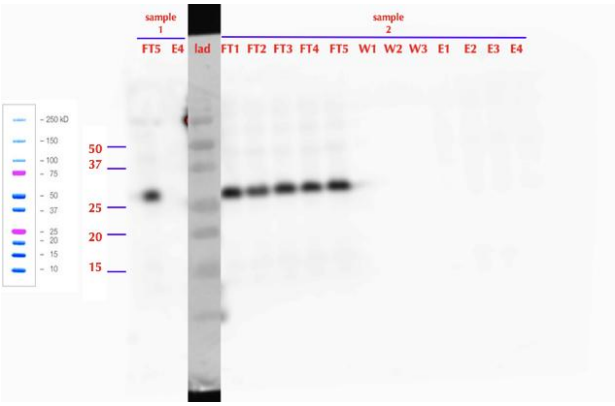
Coomassie blue

2016-07-29 mlrA ecoli, second exp.tif




Western blot

20160729_mlrA ecoli, second 2, for benchling.jpg




Based on the gel (also results from 28.7.) it appears that there might have been some protein eluted above 37 kDa marker, but based on the WB all of our protein came out as flow through, which means that there is some other protein in the gel. Because the protein came out as flow through, it seems that the His-tag is unable to bind to the Ni-NTA column. Dziga 2012 paper about production of mlrA said that they were unable to purify the enzyme using Ni-NTA even with denaturing conditions, but still we will next try that and also without imidazole in the equilibration buffer when we load the sample to the column (equilibration with imidazole, but loading without).

# 07-27/ Samples to Viikki

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-07-27 to 2016-08-17

WEDNESDAY, 7/27

Samples and MC extract are prepared according to Matti Walhsten from Kaarina Sivonen's group.

## Materials

70 % methanol

Freeze-dried cyanobacteria

## Work flow

MC extraction

- According to MC extract protocol
    - after centrifugation the sup was really dark green, maybe a bit yellowish
    - was left to evaporate the MeOH O/N

FRIDAY, 7/29

MC extraction again

## Materials

Cyanobacteria in 70 % methanol

- 50 mg of freeze-dried cyanobacteria in 1 mL of 70 % MeOH

## Work flow

MC extraction

- According to MC extract protocol
    - After centrifugation the tubes were cooled down under running water
    - We did 2 x 1 mL extractions, after centrifugation combined the samples
    - Before evaporation step there was about 1,8 mL
    - Left in RT, in chemical hood
    - After a couple of hours, there was 1,4 mL remaining in tube, 0,6 mL $H_2O$ was added

MONDAY, 8/8

First samples to enzyme activity assay.
The aim of this experiment is just to see if there is any enzyme activity in our expressed mlrA sample. That is why we will use mlrA from the first expression (18.7.) and use only the without glycerol samples, as we have no means of quantifying the amount of enzyme in the samples or compare results from the glycerol and no glycerol samples.
Based on two papers (Dziga et al. 2012 and Edwards et al., 2008, check from drive) we will use MC-LR concentration of 1 ug/mL (estimated based on estimated MC extract concentration). We will test two enzyme concentration 1:1 which is only the supernatant sample and then 1:4 dilution which is diluted to PBS. A negative control is also required for the assays.

## Materials

MC extract from 29.7.

- we will use 1 ug/ml final concentration in the tests (according to Edwards et al. 2008, and Dziga et al., 2012 from drive)

MlrA from supernatant samples from 18.7.

- we decided not to have samples with glycerol since there is no way to quantify the amount of enzyme in the glycerol and no glycerol samples so we can't compare them and can't see if there is difference in the activity due to possible better solubility of the glycerol sample
- 1:1 and 1:4 dilutions of sup samples

## Work flow

- The MC extract from 29.7. had evaporated more -> added H2O up to 2 mL -> 50 ug/ml

**Samples**

- Made samples according to table 1

Table1

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | Sample | mlrA conc. | mlrA V | MC conc. | MC V | notes |
| 2 | 1 | 1:1 | 2 mL | - | - | neg. control |
| 3 | 2 | 1:1 | 1,96 mL | 1 ug/mL | 40 uL | |
| 4 | 3 | 1:4 | 0,49 mL | 1 ug/mL | 40 uL | + 1,47 mL PBS |

Samples for enzyme activity assay

- Samples in glass tubes
- Samples incubating at +37 °C, shaking
- Took samples (à 200 ul) at different time points according to table 2
    - Timepoint samples to -20 °C right away

Table2

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Time point | 0 h | 1 h | 2 h | 4 h | 8 h | 24 h |
| 2 | sample 1 | 10:00 | 11:00 (took 240 ul) | 11:59 | 13:59 | 18:00 | 10:00 |
| 3 | sample 2 | 10:00 | 11:00 | 11:59 | 13:59 | 18:00 | 10:00 |
| 4 | sample 3 | 10:00 | 11:00 | 11:59 | 13:59 | 18:00 | 10:00 |

Time point samples taken at what times

TUESDAY, 8/9

## Work flow

- The last samples were taken
- We took all the samples to Viikki for analysis

WEDNESDAY, 8/10

## Results & conclusions

The 0 h samples didn't show any sign of MCs in them (sample 1 shouldn't in any case). We should have had a negative control with only MC. There might be some problems with the MC extraction, so we will make a new extract and analyse that in Viikki before evaporating MeOH just to see that the extraction actually works. After that we can continue with enzyme tests.

## Work flow

- Did MC extraction again according to the protocol
    - No evaporation of MeOH -> final extract in 70% MeOH

THURSDAY, 8/11

## Results

MC extract from yesterday was analysed in Viikki with HPLC and it showed that there is MC-LR. From now on everything done with MC has to be done with glass tubes or vials and glass pipets. We will do enzyme activity samples again using MC in 70% MeOH since the final amount of MeOH in the activity reactions will be low enough so the enzyme won't be affected.

So we will do the samples again today.

## Materials

Samples:

- Sample 1: negative control: 0,1 ug/ml MC + 0,1% BSA in PBS
    - 20 ul MC
    - 10 ul 10% BSA in PBS
    - 970 ul PBS
- Sample 2: 1:1 enzyme: 0,1 ug/ml MC + 0,1% BSA + enzyme
    - 20 ul MC
    - 10 ul 10% BSA in PBS
    - 970 ul enzyme
- Sample 3: 1:10 enzyme: 0,1 ug/ml MC + 0,1% BSA + enzyme diluted in PBS
    - 20 ul MC
    - 10 ul 10% BSA in PBS
    - 100 ul enzyme
    - 870 ul PBS

10 % BSA

- 20 mg BSA
- 2 ml PBS

Enzyme from second expression (25.8., more details in 07-20/29) after cell lysis and centrifugation, we used the supernatant as it was or diluted according samples.

## Work flow

- IMPORTANT! Everything which included MC was measured/pipeted/stored using glass ware, this means that amount of MC added to samples (20 ul) isn't exact as we had to use glass pasteur pipets, also when taking samples at different time points pasteurs were used so samples were approximately 100 ul
- Made samples, added MC last
- Samples according to Table 3

Table3

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Sample | 0h | 1h | 2h | 4h | 8h | 24h |
| 2 | 1 | 13:00 | 13:55 | 15:15 | 17:15 | 21:00 | 13:20 |
| 3 | 2 | 13:00 | 13:55 | 15:15 | 17:15 | 21:00 | 13:20 |
| 4 | 3 | 13:00 | 13:55 | 15:15 | 17:15 | 21:00 | 13:20 |

Table 3: Time points when samples where taken for enzyme activity assay

- Samples were about 100 ul, taken with glass pasteur pipet, right after taking the sample 100 ul MeOH was added to inactivate the enzyme
- Samples were stored in +4 °C

FRIDAY, 8/12

## Work flow

- Last sample was taken according to Table 3
- Samples in +4 °C

MONDAY, 8/15

## Work flow

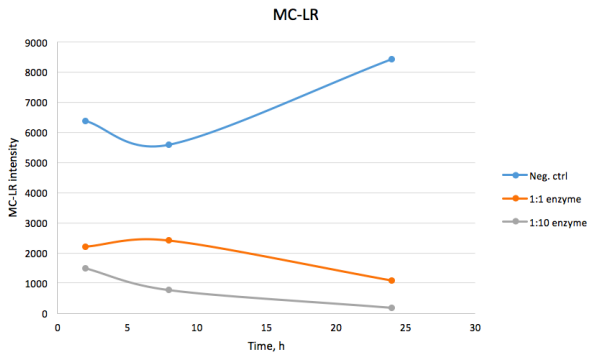
- Samples were taken to Viikki for enzyme activity assay

WEDNESDAY, 8/17

## Results and conclusions

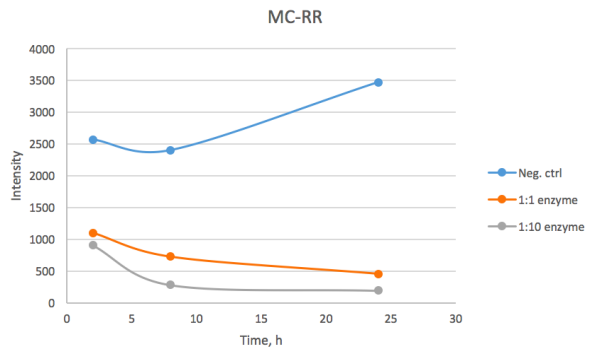
The results show the relative abundaces of MC-LR and MC-RR. The size of the peak in the excel file graphs shows the ion intensity of the MCs. Based on those, the graphs here have been plotted against time. Only time points 2h, 6h and 24h were analysed since we just wanted to check if there was any activity at all.

Näyttökuva 2016-10-02 kello 14.01.02.png




The degradation of MC-LR: The samples are the negative control, enzyme which hasn't been diluted and enzyme which has been diluted in ratio 1:10

Näyttökuva 2016-10-02 kello 14.15.09.png




The degradation of MC-RR: The samples are the negative control, enzyme which hasn't been diluted and enzyme which has been diluted in ratio 1:10

The degradation of MC-RR: The samples are the negative control, enzyme which hasn't been diluted and enzyme which has been diluted in ratio 1:10

From the graphs we can see that there is in fact activity in the enzyme samples as the amount of MC is much lower than in the negative control. Although, now we don't have the 0h samples analysed, we don't know from what amount of MCs the amounts have come down to the amounts in the these samples, but we can expect that they were somewhat same as in the negative control. Interestingly it appears that the diluted enzyme degrades the MCs faster than non-diluted. In the future experiments it will be important to make sure to take all the samples carefully and not mix anything (not that we'd have mixed something in this one) to see, if these results are reproducible, or if there has in fact been some mix up with the samples.
But since we got our positive control working, we can continue with our experiments and yeast versions, and also trying to purify the E. coli (and then the yeast) constructs further.

The mass spectrometry results can be found in the excel sheet provided here:

lcms 150816, mlrA ecoli activity.xlsx

# 08-01/10 Expression of mlrA in E. coli

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-08-01 to 2016-08-10

MONDAY, 8/1

Based on previous expression and purification of mlrA (07-20/29 Expression...) we will test to purify mlrA in two conditions: without imidazole in the loading buffer when using the Ni-NTA column, and using denaturing conditions with imidazole. These could help when the His-tag is unable to bind to the column. If these won't work we will think about purifying the enzyme using ion exchange chromatography.

## Work flow

5 mL LB-kan was inoculated with a MlrAE colony and incubated O/N at +37C.

TUESDAY, 8/2

0.5 mL of O/N culture was added to 2 x 50 mL LB-kan media.
O.D. of O/N culture was 0.675.

50 mL cultures were labelled as:
I - no imidazole
D - denaturing conditions

Incubation started at 10:30.

O.D. was monitored at regular intervals.

Table1

| | A | B | C |
|---|---|---|---|
| 1 | Time | O.D. (I) | O.D. (D) |
| 2 | 12:00 | 0.018 | 0.017 |
| 3 | 13:15 | 0.111 | 0.115 |
| 4 | 14:30 | 0.63 | 0.575 |
| 5 | 14:50 | 0.93 | 0.935 |

1 mL was taken from each culture as BI sample. Centrifuged for 10000 g for 5 min. Pellets stored at -20C.

1 mM IPTG (250 ul) was added to both cultures and cultures were incubated at 22C for 20 hours.

WEDNESDAY, 8/3

THURSDAY, 8/4

Only 'No Imidazole' samples processed today.

## Materials

1X PBS
25X PIC
Lysozyme

Equilibration buffer (EQB) - PBS, pH 7.4
Wash buffer (WB) - PBS, pH 7.4
Elution buffer (ELB) - PBS, pH 7.4

5 mL MES buffer:
20 mM MES (0.0195 g)
100 mM NaCl (0.0292 g)
5 mL ddH2O
pH adjusted to 5.0

## Work flow

50 mL culture pellet resuspended in 5 mL PBS + 1X PIC + 1/4 spatula lysozyme
Sonicated for 6 mins with same conditions as before.
Suspension divided into 2 mL tubes and centrifuged at 15000 g for 15 min at +4C.
Supernatant stored in different tube and kept on ice.

Spin-column purification

1 mL protein extract + 1 mL EQB in 2 x 2 mL tubes. Remaining supernatant stored in -20C.

Protocol from ThermoScientific was followed.
Protein mix was divided into two columns. Flow-throughs and washes for the same step were combined. Elutions were not combined.
Samples were as shown below

Table2

| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | FT1 | FT2 | FT3 | FT4 | W1 | W2 | W3 | E1 | E2 | E3 |
| 2 | | | | | | | | E1.1 | E2.1 | E3.1 |

Till FT1 collection, experiment was carried out in +4C. As the cold microcentrifuge became unavailable afterwards, remainder of centrifuging was done at RT.

SDS-PAGE

75 ul sample + 25 ul loading buffer
Heated at +70C for 5 min
15 ul loaded onto wells.

Gel run started with 80 V at 17:36.
Voltage increased to 120 V at 18:15.

Coomassie gels (protein + WB leftover) were stained with 2x used staining solution for 1.25 h.
Gels were destained O/N.

Western blotting

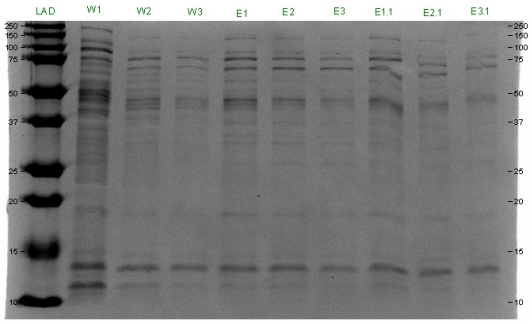
Blotting was done with TURBO settings: 25 V, 2.5 A, 7 min
Nitrocellulose membrane (NCM) was transferred to PBT-washed box and incubated at RT with blocking solution (3% BSA in PBS-T) for 1.5h.
Gel was stained with Coomassie (see above).

Blocking solution was stored back.
NCM was rinsed 3x and then washed with shaking 2 x 4 min with PBS-T. Then incubated with primary antibody O/N at +4C.

FRIDAY, 8/5

Coomassie blue staining

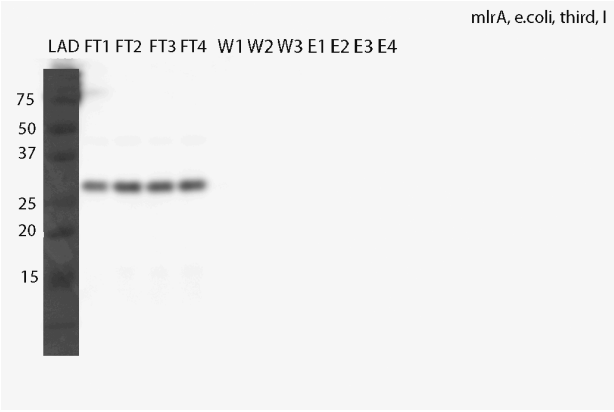
2016-08-05 mlrA ecoli, third exp.tif




The washes and the elutions did not contain any signficant amount of protein of interest. Wash 1 has a thicker band near 50 kDa. Flow-through samples were not analysed by Coomassie staining but by Western blotting (they were expected to contain the protein yet again).

Western blot

20160805_mlrA ecoli, third, overlay.tif




The MlrA was present in the flow-through once again, and the bands were of similar size as before (~28 kDa). No bands observed in washes or elutions.
Evidently, the lack of imidazole in the purification step does not improve purity of the protein.

MONDAY, 8/8

DENATURING CONDITIONS SAMPLE

## Work flow

Sample preparation

- Pellet was resuspended in 5 mL PBS + 1X protease inhibitor
- A minute amount of lysozyme was added before sonication
- Sonication was done for 5 min with usual settings
- Crude lysate was centrifuged at 15000 g for 15 min at +4C
- Supernatant and pellets were stored separately at -20C

Purification

- 2 mL supernatant + 2 mL EQB was mixed to prepare the protein extract
- Column was prepared by removing storage buffer. All centrifugations were done at 700 g for 2 min at RT. Two columns were used.
- Column was equilibrated with EQB.
- Columns were incubated with 500 ul of protein extract for 30 min on a shaker. They were then centrifuged and the flow-through was collected. This was repeated three more times. (0.5 mL x 2 x 4 = 4 mL)
- 400 ul WB was loaded into columns and centrifuged. Washes were collected. This was repeated two more times.
- 200 ul ELB was loaded into columns and centrifuged. Elutions were collected. This was repeated two more times.

For SDS-PAGE, samples were dialyzed against 8M urea (replacing the guanidine●HCl) overnight in +4C.
Dialysis was carried out in membrane tubes and closed on both ends with clips.

### TUESDAY, 8/9

Dialyzed sampes were taken into eppendorfs.

75ul sample + 25 ul 4x SDS loading buffer was mixed for SDS-PAGE. Rest of the sample was stored at -20C.

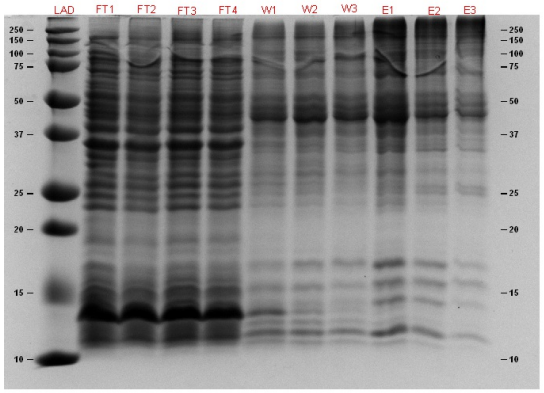
6 ul of protein ladder and 15 ul of protein samples were loaded onto wells.
SDS-PAGE was run at 80 V for 1h and later at 100V for 20 minutes.

### WEDNESDAY, 8/10

Primary antibody was collected and NCM was rinsed with PBS twice and then washed 3 times for 5 min.

NCM was incubated with secondary antibody (1% milk in PBS-T, 1:30000 dilution of RAM) for 60 min at RT.
Rinsed with PBT twice and washed for 5 min, 3 times.
2 mL detection solution was applied on NCM and incubated for 1 min, then imaged.

2016-08-10, mlrA in ecoli.tif



20160810_mlrA ecoli, third, overlay.tif

# 08-02/ Transformation and attempts of expression of mlrA (pAH13/12/14) to yeast

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-08-02 to 2016-08-18

TUESDAY, 8/2

Competent yeast cells were prepared from W303a-leu according the Competent yeast cells - LiAc transformation protocol and stored overnight in +4 °C.

WEDNESDAY, 8/3

Transformation was done according the transformation protocol for mlrA plasmids pAH11-14. As concentration of plasmids was 100ng/ul, 6ul was used in transformation reaction. 100 ul of transformation reaction was plated on minimal media -leu plates. Two parallel samples were made, other one with accidentally 1000 x too much salmon sperm.

FRIDAY, 8/5

There were many colonies on both plates. Negative control plate had no colonies. Plates were moved to fridge.

TUESDAY, 8/9

EXPERIMENT 1
Made new culture of 5 mL, minimal media -leu +2% glucose. Incubated O/N at +30 °C, shaking. (Laura)

WEDNESDAY, 8/10

EXPERIMENT 1 (CONTD.) (Snehadri's work)

## Materials

- Minimal media -leu +2% raffinose
    - 50 mL 2X MM -leu, 10 mL 20% raffinose (filtered), 40 mL $H_2O$

## Work flow

- O.D. of O/N culture was measured. MM -leu +2% glucose was used as blank.

Table1

| | A | B |
|---|---|---|
| 1 | Dilution | O.D. |
| 2 | 1 | 1.649 |
| 3 | 1/2 | 1.203 |
| 4 | 1/4 | 0.798 |
| 5 | 1/8 | 0.462 |
| 6 | 1/16 | 0.248 |

O.D. values from where a linear trend could be observed were used for calculating the required extent of dilution.

0.248*16 = 3.97
3.97*4mL = 15.88 ("O.D. of culture")

15.87/0.2 = 79

(4 mL culture to be diluted in 79 mL MM -leu +2% raffinose for refreshing, starting at O.D. of ~0.2)

O/N culture was separated in two Falcon tubes and centrifuged 3000 g, 5 min.
Each pellet was resuspended in 2 mL MM -leu +2% raf and then mixed with 28 mL MM -leu +2% raffinose.
O.D. in tubes were 0.079 and 0.362. Upon combining both cultures, O.D. of 0.226 was obtained. Final, total volume was 60 mL.
Proceeded to refreshing with raffinose at +30C for 5-6 hours [Culture 1]. Started at 11:15.

(Laura's work)

- Measured OD600 at 18:00 against freshly prepared minimal media (without Leu) with 2 % raffinose
- OD600 = 0.207, which means that the yeast hadn't grown
- Left the 60 mL culture to shake O/N at 30 °C
- Made new liquid culture [Culture 2] of 5 mL, used minimal media (without Leu) with 2 % glucose, O/N, +30 °C, shaking --> EXPERIMENT 2

## THURSDAY, 8/11

EXPERIMENT 1 (CONTD.)
[Culture 1]

O/N grown culture appeared very cloudy. O.D. values are reported below:

Table2

| | A | B |
|---|---|---|
| 1 | Dilution | O.D. |
| 2 | 1 | 2.114 |
| 3 | 1/2 | 1.68 |
| 4 | 1/4 | 1.337 |
| 5 | 1/8 | 0.88 |
| 6 | 1/16 | 0.502 |
| 7 | 1/32 | 0.269 |
| 8 | 1/64 | 0.144 |

Despite the slow growth of the yeast cells, induction with 2% galactose was carried out.
Culture was diluted 10x: 3.2 mL in 28.8 mL MM -leu +2% raffinose, to get O.D. of ~1 (ideal for pre-induction stage). O.D. was 0.765.
2% galactose was added: 1.6 mL, so final volume increased to 33.6 mL, galactose was 1.9%.
Culture was grown at +30 °C O/N, shaking.

EXPERIMENT 2

[Culture 2]

O.D. of O/N culture was measured.

Table3

| | A | B |
|---|---|---|
| 1 | Dilution | O.D. |
| 2 | 1 | 1.875 |
| 3 | 1/2 | 1.492 |
| 4 | 1/4 | 1.014 |
| 5 | 1/8 | 0.603 |
| 6 | 1/16 | 0.327 |
| 7 | 1/32 | 0.172 |

O.D. of the culture was calculated to be:

0.327*16 = 5.23
5.23*4.3 = 22.5

22.5/0.2 = 112.5 mL volume for dilution

O/N culture was separated into two Falcon tubes and centrifuged 3000 rpm, 5 min.
Pellets were washed by resuspending in 2 mL MM -leu +2% raffinose and centrifuged again.
Pellets were resuspended in 2 mL MM -leu +2% raffinose.
Suspension from both tubes were combined and put in an Erlenmeyer flask. 71 mL of MM -leu +2% raffinose was added. Total volume = 75 mL.
O.D. was 0.268.
Refreshing was started at 13:50.

After 5.5h of incubation, O.D. was measured to be 0.233. 2% galactose (3.5 mL) was added regardless, to induce protein production. (19.15 hrs)

## FRIDAY, 8/12

EXPERIMENT 1
Culture 1 was taken out of +30 °C at 11.30 and kept at +4 °C.
Centrifuged at 4500 g, 20 min, +4 °C.
Supernatant was discarded, pellet was stored in -20 °C.

EXPERIMENT 2
Culture 2 was treated the same way. It was taken out of +30 °C at 1540.
Centrifuged at 4500 g, 20 min, +4 °C.
Pellet was stored in -20 °C. 2 mL supernatant was collected in addition to the pellet for SDS-PAGE analysis. Rest of the sup was discarded.

Constructs pAH12 and pAH14 were transformed into W303a and plated (20 ul and 100 ul) on minimal media plates and left to grow in RT over weekend.

## SUNDAY, 8/14

Yeast colonies of pAH12 and pAH14 were inoculated in 5mL MM-leu + 2% glucose O/N, +30 °C, shaking.

## MONDAY, 8/15

EXPERIMENT 3

Since the raffinose refreshing is still a problem, it was decided to start a new primary culture of yeast, this time grown on MM-leu + 2% raffinose. No glucose added. Cultures from previous day were discarded.

Two colonies (for faster growth) were taken from each pAH12 and pAH14 and inoculated in 5 mL MM-leu + 2% raf and incubated at +30 °C, shaking O/N.

TUESDAY, 8/16

EXPERIMENT 3 (CONTD.)
Tubes showed very little growth after O/N incubation.

O.D. was measured at different times:

Table4

| | A | B | C |
|---|---|---|---|
| 1 | | O.D. | |
| 2 | Construct | 13:30 | 16:30 |
| 3 | pAH12 | 0.03 | 0.05 |
| 4 | pAH14 | 0.12 | 0.27 |

EXPERIMENT 4

New pre-cultures were grown on 2% raffinose, coming from two different sources. It was thought that the raffinose might be causing problems in the yeasts' growth.

## Materials

25 mL 2X SD-leu
20 mL H2O
5 mL 20% raffinose
50 mL MM-leu + 2% raf

Uracil

## Work flow

Two different SD-leu + 2% raf media were prepared:
One contained freshly prepared 2% raffinose solution.
The other contained SD-leu-ura + 2% raffinose. This came from Mari (Frey group). 3 ml/L uracil was added (30 ul in 10 mL).

The following cultures were prepared:

pAH12 in MM-leu + 2% raf (iGEM / Mari)
pAH14 in MM-leu + 2% raf (iGEM / Mari)
pJR18-Venus in MM-leu + 2% raf (iGEM / Mari) = control for yeast growth

Incubated at +30 °C, shaking 230 rpm, O/N.

WEDNESDAY, 8/17

EXPERIMENT 4 (contd.)

O.D. values of o/n grown cultures pAH12, pAH14 and pJR18-Venus

| | A | B | C |
|---|---|---|---|
| 1 | | O.D. | |
| 2 | | 09:30 | 14:40 |
| 3 | **iGEM medium** | | |
| 4 | pAH12 | 0.092 | 0.196 |
| 5 | pAH14 | 0.077 | 0.225 |
| 6 | pJR18-Venus | 0.703 | 1.423 |
| 7 | **Mari medium** | | |
| 8 | pAH12 | 0.21 | 0.546 |
| 9 | pAH14 | 0.117 | 0.334 |
| 10 | pJR18-Venus | 0.434 | 0.882 |

The pAH12- and pAH14- containing yeasts did not grow well on either medium whereas pJR18-Venus containing yeast grew better on both media, reaching desirable O.D. values. This suggests that some property of the pAH12 and pAH14 plasmids interferes with efficient yeast growth.

EXPERIMENT 5

pAH12 and pAH14 colonies were grown in five different conditions/ treatment paths:

1. 2% galactose [Gal]
2. 2% glucose → 2% galactose [Glc]
3. 2% glucose → 0.5% glucose → 2% galactose [Glc0.5]
4. 2% raffinose + leucine → 2% galactose [Leu]
5. 2% raffinose → 2% galactose [Raf]

THURSDAY, 8/18

EXPERIMENT 5 (CONTD.)

O.D. of O/N cultures, taken at 10:00

O.D. of pAH12 and pAH14 cultures for different sugars/conditions

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Culture | O.D. | Calculated O.D. | | |
| 2 | **pAH12** | | | | |
| 3 | Gal | 0.19 | | | |
| 4 | Glc | 2.75 | 6.72 | | |
| 5 | Glc0.5 | 2.67 | 6.62 | | |
| 6 | Leu | 0.49 | | | |
| 7 | Raf | 0.258 | | | |
| 8 | **pAH14** | | | | |
| 9 | Gal | 0.136 | | | |
| 10 | Glc | 2.106 | 4.51 | | |
| 11 | Glc0.5 | 2.759 | 7.232 | | |
| 12 | Leu | 1.241 | 1.976 | | |
| 13 | Raf | 0.597 | | | |

As expected, the yeasts grew very well on glucose

O.D. of high O.D. samples (for calculating actual O.D.)

pAH12

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Glc Dilution | O.D. | | Glc0.5 Dilution | O.D. |
| 2 | 1 | 2.608 | | 1 | 2.83 |
| 3 | 1/2 | 1.915 | | 1/2 | 1.962 |
| 4 | 1/4 | 1.234 | | 1/4 | 1.269 |
| 5 | 1/8 | 0.719 | | 1/8 | 0.747 |
| 6 | 1/16 | 0.397 | | 1/16 | 0.401 |
| 7 | 1/32 | 0.21 | | 1/32 | 0.207 |

pAH14

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Glc Dilution | O.D. | | Glc0.5 Dilution | O.D. | | Leu Dilutio |
| 2 | 1 | 2.23 | | 1 | 2.899 | | |
| 3 | 1/2 | 1.51 | | 1/2 | 2.082 | | 1/2 |
| 4 | 1/4 | 0.942 | | 1/4 | 1.37 | | 1/4 |
| 5 | 1/8 | 0.528 | | 1/8 | 0.825 | | 1/8 |
| 6 | 1/16 | 0.282 | | 1/16 | 0.452 | | |
| 7 | | | | 1/32 | 0.241 | | |

EXPERIMENT 6

Plasmids pAH12, pAH13, pAH14 were transformed into W303α and SS328-leu strains of *S. cerevisiae* and grown. Up until this point, only W303α strain was used. One colony was inoculated in 20 mL 1x SD-leu+2% glucose.

Rest of the experiment can be followed on 08-21/09-02 Expression and analysis of MlrA in yeast

# 08-11/18 Retransformation of pAH12-pAH14 into SS328-leu

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-08-11 to 2016-08-18

THURSDAY, 8/11

To prepare cells for transformation, *S. cerevisiae* strain SS328-leu (SS328 strain (https://www.lgcstandards-atcc.org/products/all/MYA-193.aspx?geo_country=fi) with additional deletion for leucine auxotrophy. This deletion has been selected for using ) was streaked onto YPD plates from a glycerol stock and grown for 1 day at 30 C and 3 more days in room temperature.

MONDAY, 8/15

Liquid cultures (3x5ml) from SS328-leu were prepared in YPD.  SS328 was also restreaked onto a new plate.

TUESDAY, 8/16

Dilutions from liquid culture were made to reach a starting OD of 0,5. Incubation in 30°C was continued.
Competent cells were done according the protocol and suspended in 200 ul of solution A. 100 ul was used for one transformation reaction.

Following constructs were transformed in SS328-leu according the LiAC transformation protocol:

-pAH12
-pAH13
-pAH14

All from each transformation reaction (120ul) was plated in minimal media plates.

THURSDAY, 8/18

Transformations resulted in many colonies, and also single colonies could be seen.

# 08-15/17 pAH11 in Magic Media, purification

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-08-15 to 2016-09-02

## MONDAY, 8/15

A 50 mL overnight preculture of LB + kanamycin was started with pAH11 in BL21(DE3) E. coli, according to the Magic Media protocol.

Magic media containing kanamycin was also prepared according to the Magic Media protocol and stored in +4 C.

## TUESDAY, 8/16

500 mL of magic media was inoculated with ~50 mL of preculture in a 2 L flask. Starting at 9am, culture was grown in 30 C at 220 rpm, using an air-permeable membrane to seal the flask.

500 uL of preculture was combined with 500 uL of 50 % glycerol in a cryotube to make a glycerol stock. Stock was mixed and let sit on the bench for 30 min, then transferred to -80 C.

Lysis buffer was prepared for the next day and stored at +4 C:

- 100 mM NaCl
- 3 mM $MgCl_2$
- 20 mM imidazole
- 1x protease inhibitor (amount depends on protease inhibitor brand)
- 0.1 mg/mL DNase
- 0.2 mg/mL lysozyme
- 20 mM HEPES
- pH 7.5

## WEDNESDAY, 8/17

Cells were harvested and lysed. Poured cell culture in 50 mL tubes and centrifuge at 3124 g, +4 C for 40 min. Discarded supernatant. Resuspended pellets in lysis buffer; 5 mL of buffer per 50 mL tube (for a pellet of approx. 1 g). Vortexed to break up pellet; pipette tip added in the tube to facilitate this. Removed pipette tips, combine contents of tubes so that there was ~30 mL in each tube. Tubes were left to mix; cold roller in +4 C until contents are less viscous. (~2 h). Sonication using tip sonicator; 20 % amplitude, 3 minutes, 5 s on/off cycling. Tubes kept in ice during sonication. Centrifuged for 90 minutes at 3124 g, +4 C. Collected supernatant.

This resulted in a total volume of 60 ml of collected supernatant. As the supernatant didn't appear entirely clear after 90 min of centrigfuging, the collected supernatant was centrifuged for an additional 15 min. A small pellet was observed, and the new supernatant was again transferred into new tubes.

Of the collected supernatant, a 20 mL sample was separated to be purified purified by FPLC (ÄKTA Pure, GE Healthcare).

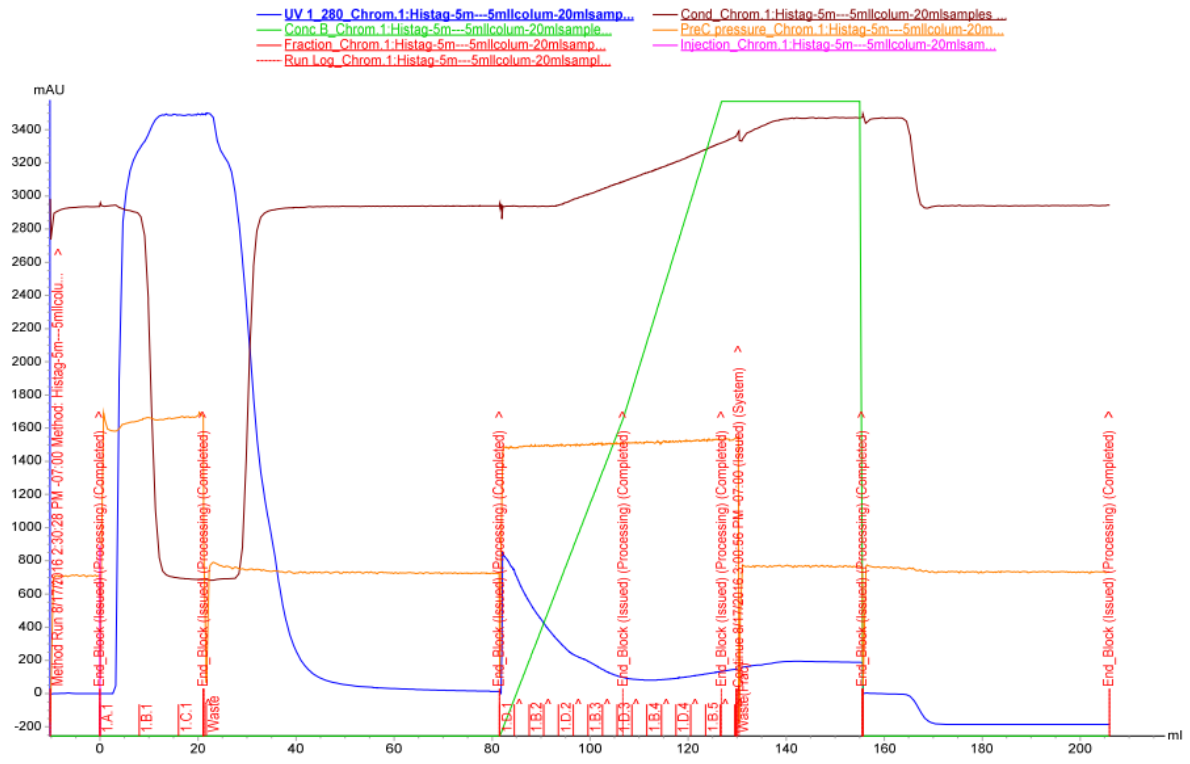
For the purification, the the following buffers were prepared, all filtered with 0.45 um HVLP filters:

- Buffer A (running buffer): imidazole 20 mM, NaH2PO4 20 mM, NaCl 500 mM, pH adjusted to 7.4
- Buffer B (elution buffer): imidazole 500 mM, NaH2PO4 20 mM, NaCl 500 mM, pH adjusted to 7.4

The sample was also filtered with a 0.45 um filter before injection into the FPLC superloop.

Purification was conduced using two stacked HisTrap FF crude 5 mL columns. UV absorbance at wavelength 280nm was used for detection.

clipboard_2016-08-19_18:34:04.png




(FPLC chromatogram)

From the FPLC chromatogram, a peak in UV absorbance is observed directly after the concentration of elution buffer starts to increase. A very small peak in UV is noted later along the increasing elution buffer gradient, around collected fractions A3 and B4. These fractions were assumed to be the most likely location of the purified, desired protein, although most likely some of it is also in the flowthrough and other fractions.

80 uL samples were collected from each fraction A1-B4 to be analyzed with SDS-PAGE and stored in -20 C.

Full details of the FPLC protocol are available in the attachment below.

protocol fplc.pdf

THURSDAY, 8/18

## Work flow

Made 3 SDS-gels, volumes of everything below

Table1

| | A | B |
|---|---|---|
| 1 | Component | Volume |
| 2 | 0.25 MTris-HCl, pH 6.8 (stacking buffer) | 2.85 ml |
| 3 | H2O | 2.4 ml |
| 4 | 40% acrylamide | 450 ul |
| 5 | 10 % APS | 45 ul |
| 6 | TEMED | 9.5 ul |

Stacking gel for 3 gels

Table2

| | A | B |
|---|---|---|
| 1 | Component | Volume |
| 2 | 1.5 M Tris-HCl, pH 8.8 (separation buffer) | 3.75 ml |
| 3 | H2O | 6.6 ml |
| 4 | 40% acrylamide | 4.7 ml |
| 5 | 10 % APS | 90 ul |
| 6 | TEMED | 18.75 ul |

Separation gel for 3 gels

SDS-PAGE samples

- all the FPLC fractions
    - 85 ul sample + 28.4 ul 4x LD
- after cell lysis spin
    - pellet diluted to the same volume it was spun down from, 70 ul sample + 23.4 ul 4x LD
    - supernatant (filtered with 0.45 um filter) 75 ul sample + 25 ul 4x LD
- samples boiled 5 min, +70 °C

Gels ran according to attached porotocol

- 2 x 2 identical gels, ones for Coomassie blue, ones for Western blotting

Coomassie blue staining

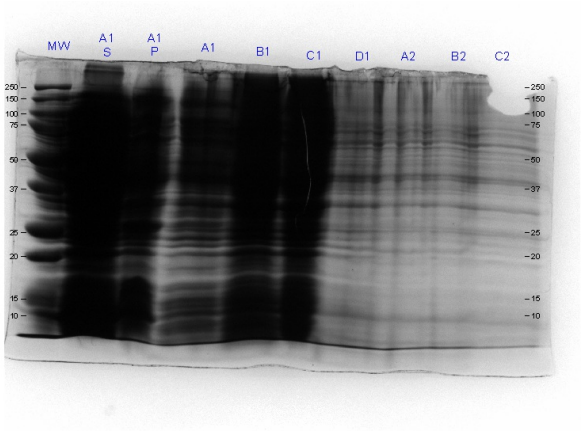
- Staining was accidentally left O/N, shaking, RT

Western blot

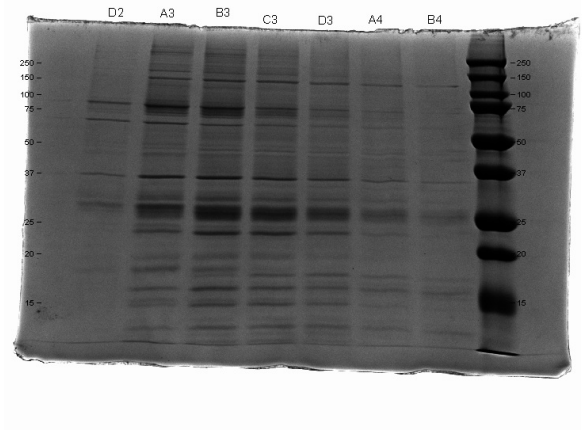
- Blotting with Standard settings
- Blocking 1,5 h, RT, shaking with 15 ml 3 % BSA in PBS-T
- Primary antibody, membranes to two 50 mL falcons, proteins facing the inside of the tube, about 7 ml Ab solution, in +4 °C, roll over, O/N

FRIDAY, 8/19

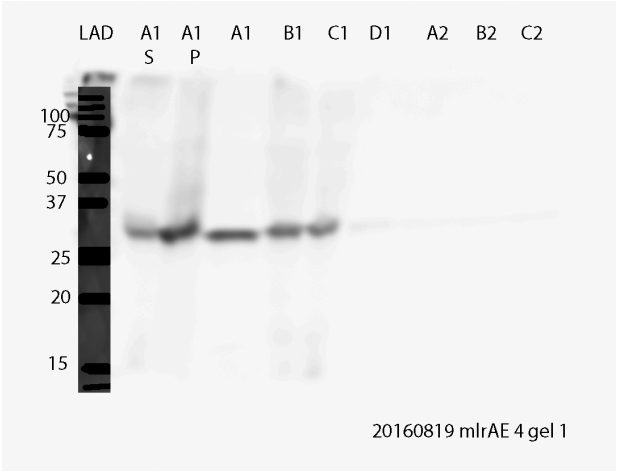
2016-08-19 pAH11 FPLC purif. gel 1, 4d destain annotated.tif



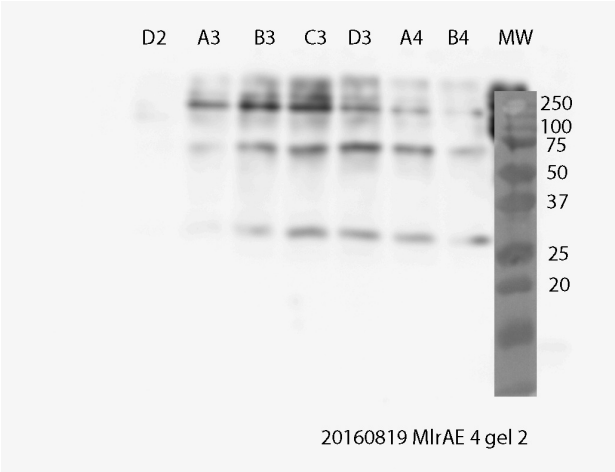

2016-08-19 pAH11 FPLC purif. gel 2, 1h destain annotated.tif




20160819_mlrA ecoli, fourth, gel 1, overlay.tif



20160819_mlrA ecoli, fourth, gel 2, overlay.tif




FRIDAY, 9/2

We analysed the activity of C3 fraction as it appears to contain the most MlrA from the elution samples. The results and how the samples were prepared are in 08-21/09-02 Expression and analysis of MlrA in yeast -entry.

# 08-21/09-02 Expression and analysis of MlrA in yeast

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-08-21 to 2016-09-02

SUNDAY, 8/21

EXPERIMENT 6, following the experiments from 08-02/ Transformation and attempts of expression of mlrA (pAH13/12/14) to yeast

Colonies of pAH12, pAH13 and pAH14 transformed in *S. cerevisiae* strains W303α and SS328-leu were inoculated in 20 mL SD- medium - leucine + 2% glucose and incubated at +30°C, shaking, O/N. The cultures were grown in 250 mL flasks. Incubation started at 16:00.

MONDAY, 8/22

O.D. of o/n cultures was measured.

pAH12

| | A | B | C | D |
|---|---|---|---|---|
| 1 | **W303α** | | **SS328-leu** | |
| 2 | Dilution | O.D. | Dilution | O.D. |
| 3 | 1 | 1.359 | 1 | 1.008 |
| 4 | 1/2 | 0.955 | 1/2 | 0.592 |
| 5 | 1/4 | 0.563 | 1/4 | 0.327 |
| 6 | 1/8 | 0.305 | 1/8 | 0.175 |
| 7 | 1/16 | 0.159 | | |

pAH13

| | A | B | C | D |
|---|---|---|---|---|
| 1 | **W303α** | | **SS328-leu** | |
| 2 | Dilution | O.D. | Dilution | O.D. |
| 3 | 1 | 1.581 | 1 | 1.56 |
| 4 | 1/2 | 1.122 | 1/2 | 1.149 |
| 5 | 1/4 | 0.695 | 1/4 | 0.712 |
| 6 | 1/8 | 0.386 | 1/8 | 0.397 |
| 7 | 1/16 | 0.201 | 1/16 | 0.211 |

pAH14

| | A | B | C | D |
|---|---|---|---|---|
| 1 | **W303α** | | **SS328-leu** | |
| 2 | Dilution | O.D. | Dilution | O.D. |
| 3 | 1 | 0.615 | 1 | 1.224 |
| 4 | 1/2 | 0.365 | 1/2 | 0.971 |
| 5 | 1/4 | 0.2 | 1/4 | 0.575 |
| 6 | 1/8 | 0.102 | 1/8 | 0.314 |
| 7 | | | 1/16 | 0.164 |

Calculated O.D. of cultures

Table7

| | A | B | C |
|---|---|---|---|
| 1 | Culture | O.D. | Volume to dilute to (for an O.D. of 0.5 from a 5 mL pellet ) (mL) |
| 2 | pAH12 W303α | 2.544 | 25.44 |
| 3 | pAH12 SS328-leu | 1.4 | 14 |
| 4 | pAH13 W303α | 3.216 | 32.16 |
| 5 | pAH13 SS328-leu | 3.376 | 33.76 |
| 6 | pAH14 W303α | 0.816 | 8.16 |
| 7 | pAH14 SS328-leu | 2.624 | 26.24 |

5 mL of culture was put in separate tubes [3 plasmids x 2 strains x 3 sugar conditions = 18 tubes] and centrifuged 3000g, 5 min, RT.

O.D. of diluted pellets are as follows:

Table8

| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | **2% galactose** | | | **0.5% glucose** | | | **2% raffinose** | | |
| 2 | **Strain** | Culture | O.D. | Volume(mL) | Culture | O.D. | Volume (mL) | Culture | O.D. | Volume (ml |
| 3 | W303α | pAH12 | 0.664 | 25 | pAH12 | 0.494 | 31.5 | pAH12 | 0.369 | 4 |
| 4 | | pAH13 | 0.629 | 32.5 | pAH13 | 0.507 | 46.5 | pAH13 | 0.448 | |
| 5 | | pAH14 | 0.356 | 8.15 | pAH14 | 0.65 | 16.5 | pAH14 | 0.359 | 1 |
| 6 | | | | | | | | | | |
| 7 | SS328-leu | pAH12 | 0.676 | 14 | pAH12 | 0.449 | 24 | pAH12 | 0.423 | |
| 8 | | pAH13 | 0.588 | 32.5 | pAH13 | 0.502 | 39 | pAH13 | - | - |
| 9 | | pAH14 | 0.615 | 25 | pAH14 | 0.565 | 31.5 | pAH14 | 0.429 | |

Table1

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | **0.5% glucose** | | | | **2% raffinose** | | | |
| 2 | **Strain** | Culture | O.D. (undiluted) | Volume (mL) | **Volume of 40% galactose added (mL)** | Culture | O.D. (undiluted) | Volume (mL) | **Volume of 4( galactose added (mL)** |
| 3 | W303α | pAH12 | 1.841 | 31.5 | 1.65 | pAH12 | 0.358 | 46.5 | 2 |
| 4 | | pAH13 | 1.798 | 46.5 | 2.45 | pAH13 | 0.448 | 49 | |
| 5 | | pAH14 | 1.672 | 16.5 | 0.868 | pAH14 | 0.359 | 16.5 | 0.8 |
| 6 | | | | | | | | | |
| 7 | SS328-leu | pAH12 | 1.508 | 24 | 1.3 | pAH12 | 0.423 | 24 | 1 |
| 8 | | pAH13 | 1.464 | 39 | 2.1 | pAH13 | - | - | |
| 9 | | pAH14 | 1.649 | 31.5 | 1.65 | pAH14 | 0.429 | 39 | 2 |

*Dilution refers to the dilution series for calculating actual O.D. of culture.

Volumes of galactose were calculated as such:

$40 \times V_1 = 2 \times (V_2 + V_1)$
$40V_1 = 2V_2 + 2V_1$
$38V_1 = 2V_2$
$V_1 = 2V_2/38$
$V_1 = V_2/19$

V1 = volume of 40% galactose
V2 = volume of media

TUESDAY, 8/23

AI sample (1 mL) taken from both cultures - pellet and supernatant.

Cultures' incubation stopped at:
14:00 - Gal samples
16:00 - Glc0.5 samples
20:00 - Raf samples

Cultures centrifuged at 3200 g for 40 min at +4 °C. Pellets and supernatants stored at -20 °C.

Samples

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 12 | 12 | 12 | 12 | 13 | 13 | 13 | 13 | 14 | 14 | 14 |
| 2 | W303 | W303 | SS328-leu | SS328-leu | W303 | W303 | SS328-leu | SS328-leu | W303 | W303 | SS328-leu |
| 3 | pellet | sup | pellet | sup | pellet | sup | pellet | sup | pellet | sup | pellet |

WEDNESDAY, 8/24

## Work flow

We decided to try the yeast cell lysis and analysis only with one strain and one condition, but with all the constructs. We concluded that the best one to try is the SS328 yeast which is refreshed with 0.5 % glucose, since all the other yeast expressions have been done in this strain and refreshing with 0.5 % provided the biggest growths.
Construct were pAH12(-MlrAY), pAH13(-MlrAYAlpha) and pAH14(-MlrAY3Alpha)

- Pellets were quite small, smallest in pAH14, slightly bigger in pAH12 and about three times the others was pAH13. Also, pAH13 was very pink, pAH14 faintly pink and pAH12 normal yeast colour
    - The pinknesh is due to some quite normal mutation in the SS328 strain according to someone in our lab

Yeast cell lysis according to yeast cell lysis protocol

- H2O to resuspend the pellets was estimated so that there was at least 1 mL and then according to size
    - pAH12 in 1 mL
    - pAH13 in 3 mL
    - pAH14 in 1 mL
- for lysis the washed pellets were resuspended in same amounts as in H2O, following volumes are the overall volumes of pellet/lysis buffer solutions
    - pAH12 1,22 mL
    - pAH13 3,5 mL
    - pAH14 1,2 mL
- after cell lysis, but before spin Crude Lysate (CL) samples (à 45 ul) were taken for SDS-PAGE
- after spin sup was and the glass beads scooped out as much as possible without touching the pellet
- pellets were resuspended in same amounts as they were spinned from
- samples from supernatant 2 (S2) and pellet (P2) à 45 ul were taken for SDS-PAGE

SDS-PAGE samples

- samples were
    - supernatant 1 (S1) which was the sup after cultures were spun down
    - crude lysate (CL) after lysis

    - sup 2 (S2) after lysis and spin
    - pellet 2 (P2) after lysis and spin
- all samples were prepared as follows
    - 45 ul sample + 15 ul 4 x LD
    - boiled 5 min at 70 °C

SDS-PAGE
- two identical gels
- gel was run normally, started with 80 V for about an hour, then to 120 V
- one gel to coomassie blue staining, one for western blot

Coomassie blue
- stain 2 h, shaking, RT
- destain O/N, shaking, RT

Western blot
- blotting using BioRad's TURBO machine, standard 30 min protocol
- membrane rinsed with PBS-T
- blocking with 3 % BSA in PBS-T, 1,5 h, RT, shaking
- primary antibody ON, RT, shaking

Enzyme activity
- According to enzyme activity sample protocol
- We did parallel also enzyme activity samples from the S2 samples
    - 485 ul enzyme sample + 5 ul 10 % BSA + 10 ul MC (not sure if there is any)
    - negative control was the same except instead of enzyme sample there was just PBS
- 0h, 1h, 3h, 5,5h samples were taken à 70 ul + 70 ul MeOH
- left O/N to +37 °C shaker

THURSDAY, 8/25

## Work flow

Coomassie blue
- Imaged gel, check results
- put gel back to destain if the background would became a bit clearer

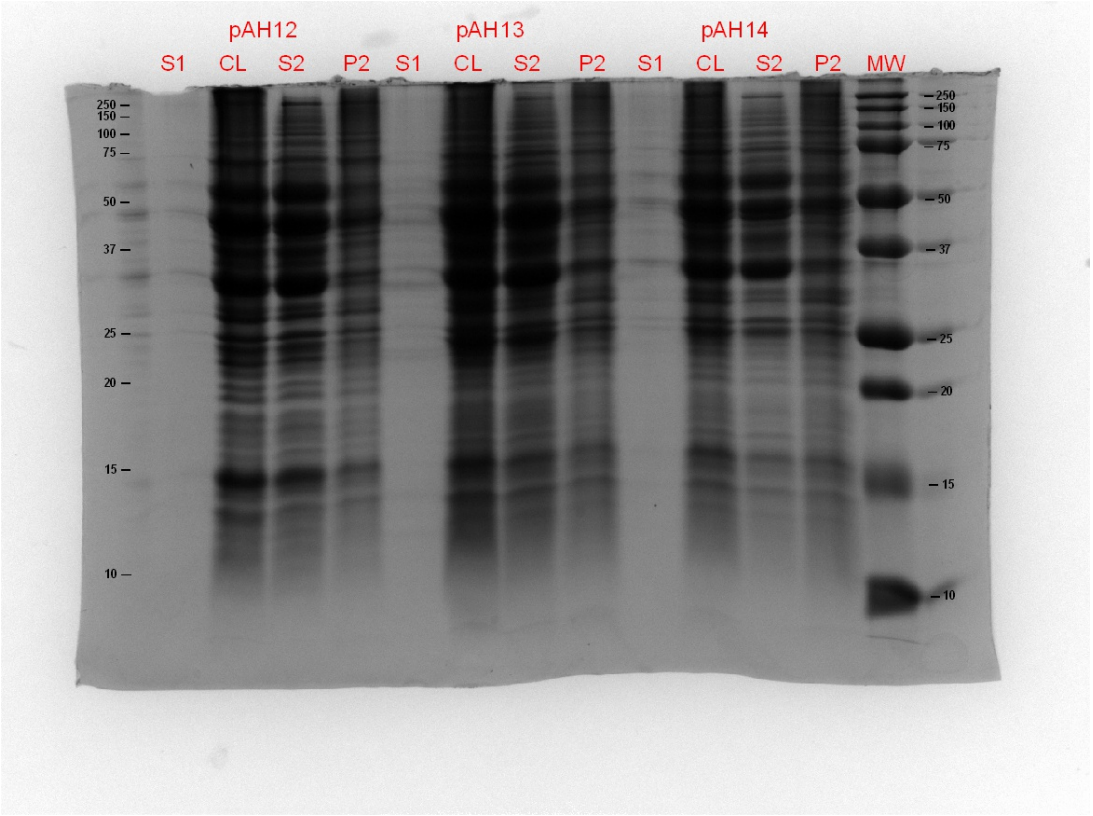
Western blot
- Primary antibody was forgotten to RT, should've been at +4 °C, so we permorfed long wash: 2 x rinse, 4 x 10 min wash at shaking with PBS-T, RT
- Secondary antibody (1:30 000 dilution in 1 % milk in PBS-T, 15 mL), 1 h, RT, shaking
- Normal wash: 2 x rinse, 3 x 5 min shaking, RT, in PBS-T
- Reagents to make the chemiluminescense 1 mL + 1 mL
- Imaging, see results

Enzyme activity samples
- The samples had evaporated so there wasn't anything to get

## Results and discussion

Coomassie blue

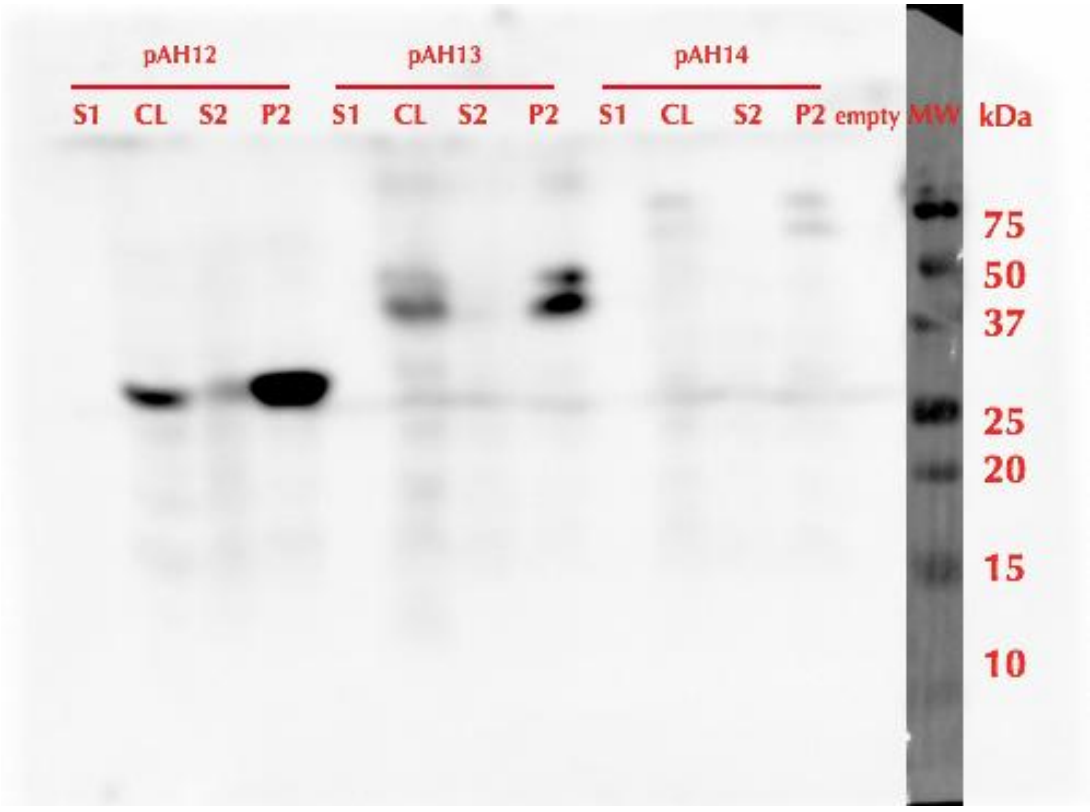
2016-08-25 mlrA in yeast, ss328, 0,5 Glc refresh, ANNOTATED.tif




SDS-PAGE of three MlrA Yeast constructs

Western blot

20160825_mlrA in yeast, western blot.jpg




Western blot of yeast expressed MlrA constructs

The theoretical molecular weights for different contructs are the following:

- pAH12 = 37,4 kDa
- pAH13 = 46,7 kDa
- pAH14 = 61,4 kDa

How ever, it seems that in the western blot the pAH12 bands are about 28 kDa, the same as they should be in bacteria because of the proteases. In pAH13 and pAH14 there are double band visible. In pAH13 it might be that the upper band is MlrA + Alpha mating factor and in the lower band the Alpha factor has been cleaved leaving only 37 kDa sized MlrA. In pAH14 it could also be that the upper band represents the whole construct, MlrA + 3 alpha factors, and the lower band is the construct that has been cleaved. Based on the WB the use of S2 in the enzyme activity samples wasn't good as it seems that there isn't anything there. We will do the samples again using the pellet 2 fractions this time.

MONDAY, 8/29

## Work flow

Enzyme activity samples from P2 samples and also from purified E. coli construct (FPLC fraction C3 from 08-15/18) were made according to enzyme activity preparation protocol

- 1 mL reactions were made: 970 ul enzyme + 20 ul 50 mg/L MC extract + 10 ul  10 % BSA
- 100 ul samples were taken and inactivated with 100 ul MeOH
- different timepoint samples were taken according to table
- samples to +37 °C shaking at 10:22

Table2

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | Sample | 0 h | 1 h | 2 h | 6 h | 24 h |
| 2 | pAH12 | 10:12 | | | 16:29 | |
| 3 | pAH13 | 10:14 | | | 16:31 | |
| 4 | pAH14 | 10:15 | | | 16:33 | |
| 5 | pAH11 | 10:16 | | | 16:35 | |
| 6 | neg. | 10:18 | | | 16:36 | |

TUESDAY, 8/30

## Work flow

Took the last sample (24 h)

Samples were taken to Viikki for enzyme activity assay

FRIDAY, 9/2

We got enzyme activity results from Viikki (actually yesterday)

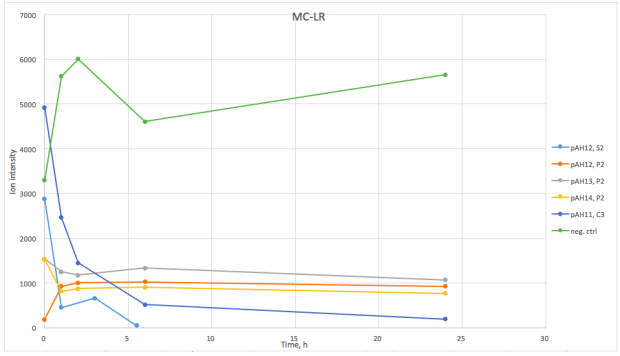
## Results and conclusions

The raw data can be found from the attached excel file. There are the graphs of the LCMS analysis of the MC-LR and MC-RR amounts from different samples, and based on them the two graphs have been drawn.

LCMS, 310816, enzyme activity.xlsx

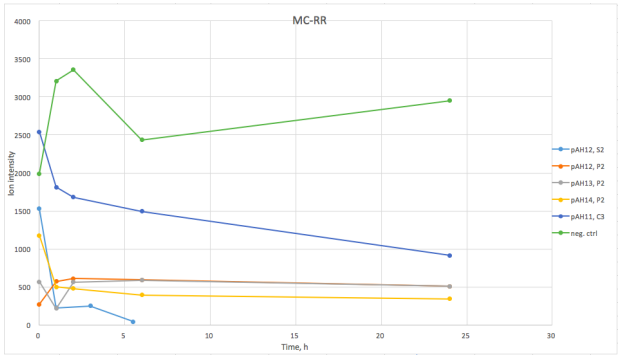
Results from pAH11, pAH12, pAH13, pAH14 and neg. ctrl enzyme activity assay

MC-LR




Enzyme activity assay results; the degradation of MC-LR

MC-RR




Enzyme activity assay results; the degradation of MC-RR

Based on the enzyme activity assays, it seems that the pellet fractions of the expressed MlrA in yeast are not active. The S2 sample of the pAH12 might have some activity, but it needs to be done properly again. But atleast the purified pAH11 from E. coli has good activity.

As pAH13 and pAH14 should be in the membranes, they should appear in the pellet fraction as they do. It might be that the enzymes are in the membranes and are active, but, it might also mean that they are denaturated and do not function. The enzyme activity test points towards the former option as it seems that the levels of MC drop very fast during the first hour of incubation. This also suggests that the enzyme should be purified in the membranes as it is very active in them. Next time we should have dilutions of this to see how fast the enzyme really degrades the toxin and also have more time points in the beginning of the experiment.

pAH12 also localized mostly into the pellet fraction, but there is also some in the soluble supernatant fraction, and as we planned, pAH12 should be soluble. It seems that the supernatant fraction has some activity, but the graph is not as nice as in the E. coli version, and the samples should be done again for the supernatant sample. The pellet sample showed the same results as the pAH13 and pAH14.

The E. coli (pAH11) MlrA from the FPLC purified fraction shows good activity, and it seems that it has greater affinity towards MC-LR compared to MC-RR.

The negative sample has a drop in the 6h sample, and also the 0h sample shows quite low readings compared to the rest of the timepoints. This might be due to the inaccuracy of using a pasteur pipet to take the samples: some have less enzyme reaction in them but all are inactivated by the same amount of MeOH, which makes some samples less concentrated than others, even though they should be tha same. In the future this could be avoided by parallel samples of the same reaction. Also right in the beginning the samples might not have been mixed thoroughly before taking the 0h sample, resulting in low concentrations of MC.

# 09-02 MlrA expression in yeast

**Project:** Microcystinase (MlrA)
**Authors:** Laura Laiho
**Dates:** 2016-09-01 to 2016-10-03

THURSDAY, 9/1

Colonies of pAH12, pAH13 and pAH14 from SS328-leu plates were restreaked on new plates and incubated at RT over weekend.

FRIDAY, 9/2

## Materials

- 2X SD-leu (50 mL) pH 5.8, filter sterilized
  - 0.67g Yeast nitrogen base
  - 0.2 g amino acid (-leu) drop-out
- 1X SD-leu + 2% glucose (100 mL)
  - 50 mL 2X SD-leu
  - 10 mL 20% glucose
  - 40 mL $H_2O$

## Workflow

Filter sterilized SD-leu media containing 2% glucose [100 mL] was prepared.
Four cultures à 5 mL media for each construct were prepared and incubated at +30C, shaking o/n. Incubation started at 19:20.

TUESDAY, 9/6

## Materials

- 1X SD-leu + 0.5% glucose - via filter-sterilization
  - 250 mL 2X SD-leu
    - 3.35 g yeast nitrogen base - amino acids
    - 1 g amino acid drop-out mix
    - pH adjusted to 5.8
    - made up to 250 mL with $H_2O$
  - 12.5 mL 20% glucose
  - 237.5 mL $H_2O$
- 40% galactose
- 2X SD-leu (500 mL prepared and sent to autoclave)
  - 6.7 g yeast nitrogen base -AA
  - 2 g AA drop-out mix
  - pH 5.8

## Workflow

pAH12 and pAH13 were used in the experiment. Reasons:
- pAH12 gave some positive result in the LC-MS from previous experiment.
- pAH13 chosen as it has at least a secretion signal and we can study whether there is a change in the localization of the protein.

O/N cultures were combined and O.D. was measured.

O.D. for pAH12 and pAH13 were 8.32/mL and 6.14/mL.

Both cultures were refreshed in SD-leu +0.5% glucose.
pAH12: 16.3 mL culture was diluted in total 500 mL media. O.D. after dilution was 0.184. Refreshing was done from 13:50 to 19:40.
O.D. before induction was 0.784.

pAH13: 12.0 mL culture was diluted in total 500 mL media. O.D. after dilution was 0.199. Refreshing was done from 14:45 to 20:35. O.D. before induction was 0.818.

26.3 mL 40% glucose was added to both cultures to induce protein production

WEDNESDAY, 9/7

16:00 pAH13 culture was taken out of incubator and centrifuged in 10x 50 mL tubes.
Centrifuging at 3200 g for 40 min at +4C.
Pellets and 200 mL of supernatant were stored in -20C.

17:00 pAH12 culture was treated the same way.
Pellets and 200 mL of supernatant were stored in -20C.

MONDAY, 9/12

Cell lysis

## Materials

- Lysis buffer (LB)
  - 1X PBS, ice-cold
  - 1 mM DTT
  - 10 mM PMSF
  - 1X PIC (EDTA-free)
- 100 mM PMSF
  - 0.087 g PMSF in 5 mL 99.5% ethanol
- 25X protease inhibitor (PIC)
  - One tablet in 4 mL 1X PBS. Tubes covered with aluminium foil.

## Work flow

- pAH12 and pAH13 pellets were weighed: 0.63g and 1.05 g respectively
- Pellets were resuspended in 2 mL (pAH12) and 3 mL (pAH13) LB
- Slurry was divided into 2 mL tubes, with 1 mL maximum volume. Equal volume of acid-washed glass beads were added (turned out to be of more volume)
- Samples were vortexed at full speed for 1 min and then cooled down for 1 min. This was repeated for 10 more times.
- Broken cells were transferred to new tube. Beads were washed with LB to get more cells. Beads were avoided during transfer.
- 10 mM PMSF (250 ul/pAH12 and 350ul/pAH13) was added.
- Slurry was divided into 2 mL tubes and centrifuged 17 000 g for 10 min at +4C.
- Supernatant was separated from pellet, and both were stored at -20C.

TUESDAY, 9/13

Refolding of the possibly denaturated soluble proteins from the pellet samples after cell lysis

## Materials

- Solubilization buffer (SB)
  - 50 mM Tris-HCl, pH 7.4
  - 4 M urea
- Refolding buffer (RB)

- 10 % (v/v) glycerol
- 0.1 mM EDTA
- 20 mM HEPES pH 7.4

## Work flow

- Resuspended pellets from yesterday (2 for each construct) in 1.5 mL of SB
  - combined samples so 3 mL of each
- Washed with sonication, 4 min, 5 sec on/ 5 sec off, 30 % amp
- Centrifugation 12 000 g, 10 min, RT
- Collected supernatant
- Repeated still three times (four times all together)
  - Final volume of the supernatant about 12 mL, a bit cloudy, but not much
- Incubation of the supernatant, 1h, +37 °C (no shaking)
- sample put to dialysis according to refolding protocol

WEDNESDAY, 9/14

Dialysis bags had leaked, started new refolding according to refolding protocol. Samples from pAH12 & pAH13 pellets after cell lysis from 12.9.

- dialysis left ON according to protocol

THURSDAY, 9/15

Dialysed proteins were taken out.
Volumes were:
pAH12 - 7 mL
pAH13 - 4.5 mL

They were further concentrated at 2000 g, +4C, 20 min.
Concentrated volumes were:
pAH12 - 4 mL
pAH13 - 2.3 mL

SDS-PAGE analysis

Loading: 45 ul sample + 15 ul loading dye, exception - P2: 15 ul sample + 7.5 ul $H_2O$ + 7.5 ul loading dye

S1 - growth media
S2 - supernatant after cell lysis
P2 - pellet from cell lysis
S3 - renatured protein
P3 - cell walls + plasma membrane

After electrophoresis, WB gel was put in transferring buffer for a few minutes with the other layers (Whatman paper and NCM).

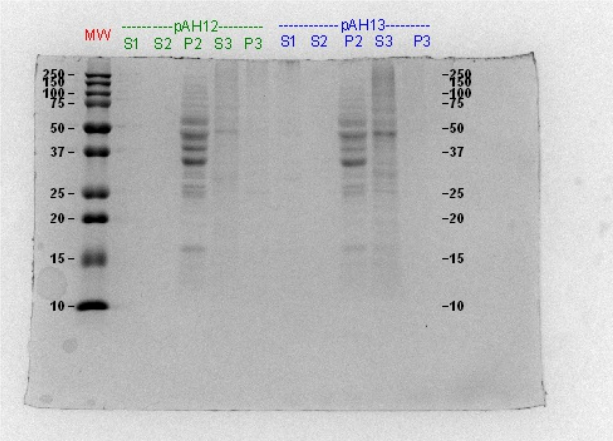
Blotting: problem with current, did not go up to 2.5A. Blotting was done double-time (1 h) to compensate for this. Bad idea - lots of heat generated and the gel was almost burnt.

NCM was washed with PBS-T twice and blocked with 3% BSA in PBS-T for 1.5 h.
It was washed 2x and rinsed 3x for 5 min with PBS-T and then incubated with primary antibody overnight at +4C.

FRIDAY, 9/16

SDS-PAGE

2016-09-16 mlrAY-Ya first exp.tif




Western blotting

- Primary antibody was poured back into tube.
- NCM was rinsed and washed with PBS-T.
- Incubation with secondary antibody in 1% milk in PBS-T for 1h, RT, shaking.
- NCM was rinsed and washed with PBS-T.
- There wasn't enough peroxidase component of ECL substrate!! - NCM was dried and stored at +4C over the weekend.

MONDAY, 9/19

Ultracentrifuge at VTT

## Materials

1X PBS buffer with 5% glycerol

## Workflow

Transferred ~2 mL of sample into UCF tubes.
Balanced with water. Mass same up to 0.01 g.
Centrifuged at 48000 RPM (160 000 g) for 1 h at +4C.
(Rotor max speed was 64 000 RPM)

Supernatant removed (but stored at -20C).
Pellets resuspended in 300 ul of buffer.

Microsome suspension homogenized with douncer in eppendorf tubes.
Absorbance at 280 nm was measured with Nanodrop.

pAH12
21.94 mg/mL
22.22 mg/mL
22.6 mg/mL

Average = 22.3 mg/mL

pAH13

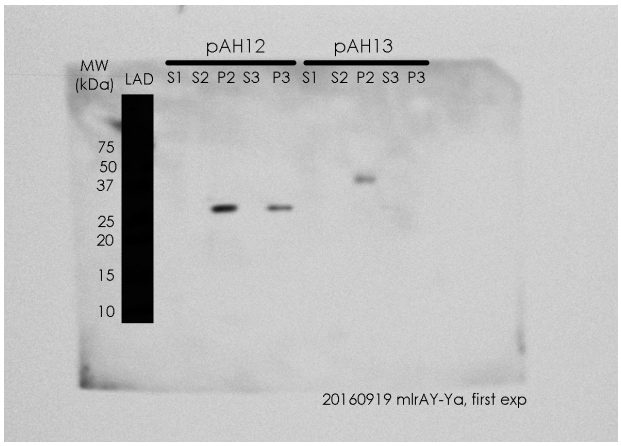
14.82 mg/mL
14.88 mg/mL
14.81 mg/mL

Average = 14.84 mg/mL

Western blotting

NCM from last week was moistened with PBS-T and then incubated with ECL substrate for 1 min.
Imaged with LAS-3000.

20160919_mlrAY-Ya overlay.tif




TUESDAY, 9/20

Cell lysis

Same as before (see 5.9.)

This time, 5 ml of supernatant was obtained from the lysed cells of both constructs.

WEDNESDAY, 9/21

Workflow

- Pellets from cell lysis were resuspended in 1.5 mL of 50 mM Tris-HCl, pH 7.4, 4 M urea.
- Samples were sonicated for 4 min at 30% amplitude, with 5 sec on/off cycles.
- Supernatant was collected in a new tube.
- Steps 1-3 were repeated three more times and the supernatant was collected in the same tube.
- Supernatant was incubated at +37C for 1h, shaking at 230 rpm.
- 1-2 mL of 8M urea was added to both samples to get clear solution. Final volumes were: pAH12 - 12 mL, pAH13 - 13 mL.
- Clear samples were incubated at +37C for 2 h 15 min (instead of 1h, they were forgotten there).
- Samples were dialyzed at 1:8 ratio against 20 mM HEPES pH 7.4, 10% v/v glycerol, 0.1 mM EDTA [25 mL:200 mL] at +4C overnight.

THURSDAY, 9/22

Workflow

- Samples were collected from tubes and concentrated by centrifuge at 2000 g, 20 min, +4C.

- pAH12: 10 mL, concentration after cfg = 1 mg/mL
- pAH13: 8 mL, conc. = 2 mg/mL

- Samples were dialysed against same buffer [800 mL] at a ratio of 1:44 at +4C overnight

FRIDAY, 9/23

Dialysis samples were taken out.
Volumes: pAH12 - 8 mL, pAH13 - 5 mL

Samples concentrated with filter columns
pAH12 - 7.5 mL, 0.912 mg/mL
pAH13 - 4.5 mL, 2.05 mg/mL

SS328-leu o/n cultures were centrifuged and lysed. Crude lysate was used as negative control in SDS-PAGE analysis.

SDS-PAGE and WB

Samples were loaded in the same order on both gels.
Order can be seen in gel images.

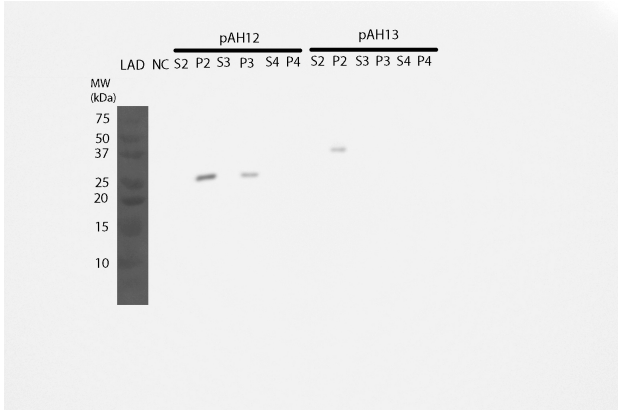
SATURDAY, 9/24

Primary antibody was poured away from NCM and stored at +4C. USED NINE TIMES SO FAR.
NCM was rinsed 2x and then washed 3x for 10 min with PBS-T.
NCM was incubated in 15 ml of secondary antibody (1:30000) in 1% milk in PBS-T at RT for 1h.
Secondary antibody was poured away
2 mL of ECL substrate was poured on membrane and incubated at RT for 1 min.
Substrate was removed with whatman paper -- maybe too soon or too much removed...
2 mL of ECL substrate was poured on NCM again, and this time after the 1 min, the substrate was not removed.

NCM was analysed by Fujifilm LAS 3000 imager.
Exposure time: 10 sec. Last image taken at image 26.

For the overlay, digitized image was edited to have full contrast, to further highlight the ladder bands.

20160923_mlrAY-Ya 2nd overlay.tif

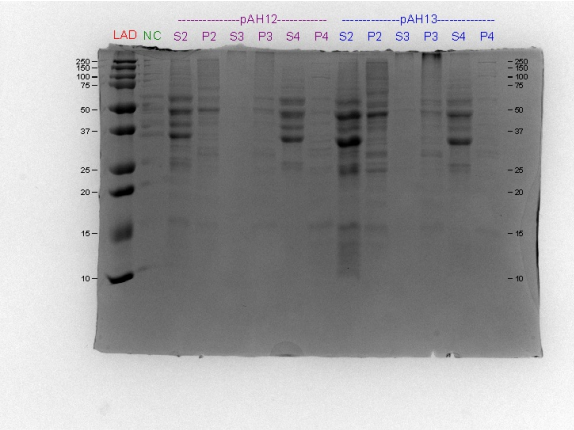



Band of ~28 kDa observed in P2 (post-cell lysis) and P3 (pre-renaturation) samples of pAH12.
Band of ~37 kDa observed in P2 sample of pAH13.
For once, very low signal was observed from ladder bands (hidden here by the superimposed ladder, but there was only one band).

Coomassie stained gels are below:

Main gel

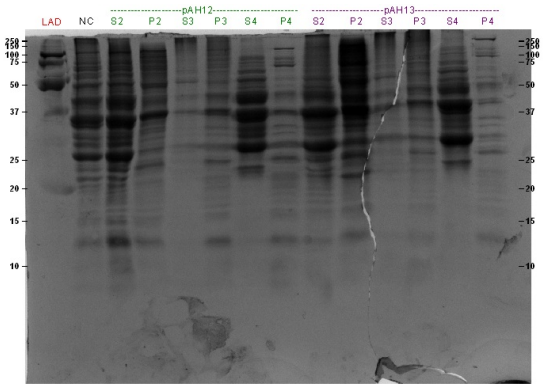
2016-09-24 mlrAY-Ya 2nd exp.tif




Some lanes do not contain any protein.
Most likely explanation is that this gel was destained twice - the second time being 1h after the first destaining.
Destaining at roughly 15.00h on the previous day till 12.00 today = 21h destaining.

WB leftover gel

2016-09-24 mlrAY-Ya 2nd exp WB leftover.tif




This gel surprisingly has more protein bands than the normal gel. Once again, this could be due to the fact that this gel was destained only once and was destained for 20h (not a significant time difference).
S3 of both constructs has low protein concentration - suggesting that protein was lost during dialysis as a result of leakage.

MONDAY, 9/26

## Workflow

Same SDS-PAGE samples were run on 12.5% gels (freshly made today).

After 1h it was noticed that the proteins were not running down the gel. The fault was attributed to the electrophoresis chamber.
SDS-PAGE proceeded fine upon changing the chamber.

Western blotting

Blotting:
LIST→ BIO-RAD → 1 MINI-GEL → EDIT → 25V constant, 2.5 A limit, 30 min.
Still, current was around 0.6A.
At the end, the transfer seemed to have succeeded well.

NCM transferred to box and blocked with 3% BSA in PBS-T for 1h, shaking. No washing between blotting and blocking.
NCM rinsed 2x and washed 3x for 8 min with PBS-T. -- maybe this long wash was unnecessary?
NCM was incubated with 15 mL primary antibody in 3% BSA in PBS-T and incubated at +4C, shaking overnight. [started at 21.45]

Gel was Coomassie-stained for 1h 15 min.
Destaining from 2105.

Coomassie
Destaining of main gel was started at 2030. Done only once.

TUESDAY, 9/27

## Workflow

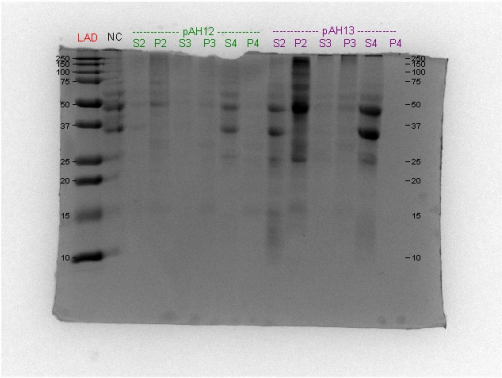
MC extraction

Did this in Viikki.

- (Matti) Measured 253 mg of freeze dried cyanobacteria
- Resuspended in 5 ml 70% MeOH
- incubated 30 min in +80 °C
- vortex 5 min
- incubated 30 min in +80 °C
- centrifuge 12 000 g, 5 min
- collected supernatant

SDS-PAGE

2016-09-27 mlrAY-Ya 2nd exp-2.tif

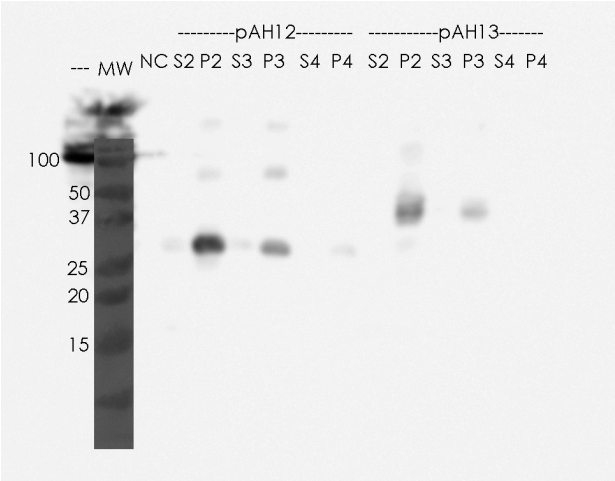



Low protein bands observed in both gels.

Western blotting

- Primary antibody was poured back into tube and small amount of $NaN_3$ was added. Tube was stored at +4C.
- NCM was rinsed and washed 3x for 8 min with PBS-T, then incubated in secondary antibody (RAM) in 1% BSA in PBS-T for 1h at RT, shaking.
- NCM was rinsed and washed once for 5 min with PBS-T
- 2 mL detection solution was poured on NCM and incubated for 1 min.
- NCM was imaged in LAS-3000.

20160927 MlrAY-Ya WB 2nd exp-2 overlay.tif




Enzyme is present in P2 and P3 samples in both pAH12 and pAH13. Relatively higher amount in P2.
However, no more of the P2 sample is left.

Enzyme activity samples

Based on the WB we chose to have P3 samples from both constructs to the final enzyme activity measurements.
Samples according to table 1, triplicates of each

Table1

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | Sample | Construct | MC | 10% BSA | Sample dilution | Enzyme | PBS |
| 2 | 1 | neg. ctrl | 20 ul | 10 ul | - | - | 970 ul |
| 3 | 2 | neg. yeast ctrl | 20 ul | 10 ul | 1:10 | 100 ul | 870 ul |
| 4 | 3 | pAH12 | 20 ul | 10 ul | 1:10 | 100 ul | 870 ul |
| 5 | 4 | pAH12 | 20 ul | 10 ul | 1:100 | 10 ul | 960 ul |
| 6 | 5 | pAH12 | 20 ul | 10 ul | 1:1000 | 10 ul of 1:10 dilution | 960 ul |
| 7 | 6 | pAH13 | 20 ul | 10 ul | 1:10 | 100 ul | 870 ul |
| 8 | 7 | pAH13 | 20 ul | 10 ul | 1:100 | 10 ul | 960 ul |
| 9 | 8 | pAH13 | 20 ul | 10 ul | 1:1000 | 10 ul of 1:10 dilution | 960 ul |

Enzyme activity samples

The sample reaction were incubated at +37 °C
Timepoints that we used: 0h, 15 min, 30 min, 1h, 2h, 4h
100 ul of the reaction was taken at each timepoint
The samples were inactivated with 100 ul of MeOH

WEDNESDAY, 9/28

## Workflow

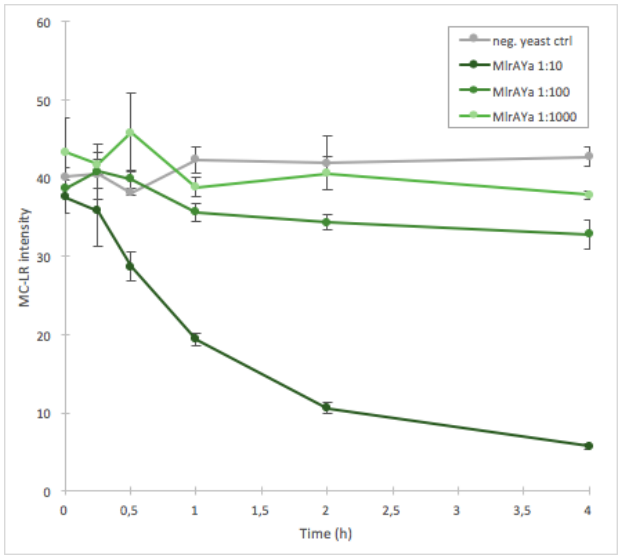
Took the enzyme activity samples to Viikki, where we first centrifuged them at 21000 g for 5 min and filtered the supernatant with 0.2 um filter. Matti will take care of the rest.

Samples 1.1 and 8.2 are missing.

## MONDAY, 10/3

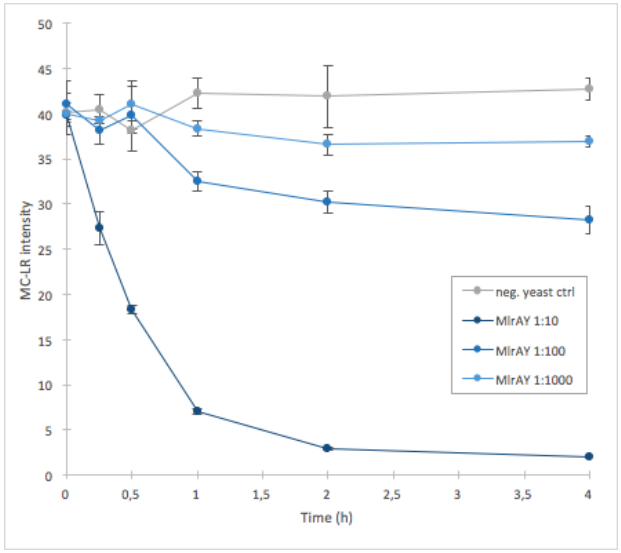
Results came back looking very nice, see pictures

Näyttökuva 2016-10-16 kello 12.58.46.png




pAH12 activity

Näyttökuva 2016-10-16 kello 12.57.04.png




pAH12 activity